MYCOREMEDIATION

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*To my beloved wife, **Julu**, a font of unconditional love and inspiration, for her support, understanding, and encouragement during the completion of this book*

*In memory of my divine **Mother** (1926–2005), who built my foundation and provided perennial blessings, unending care, and motivation throughout my life*

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PREFACE

Scientists began to use fungi and bacteria for the degradation of xenobiotic organic compounds toward the middle of the twentieth century. The use of bacteria showed fast and promising results, but research on evaluating fungi has lagged behind. This does not mean that fungi are not suitable organisms or that they function less satisfactorily than bacteria in degrading such compounds. The participation of fungi in bioremediation is now well established in all ecosystems.

Mycoremediation is one of the most complex areas in applied remediation engineering. During the past two decades, many fungal scientists and engineers have wanted to try using fungi in the degradation of organic compounds, and for those who did try using them, good results were obtained. The discovery of the value of white-rot fungi in bioremediation has brought greater success and has thus stimulated research throughout the world. A new era in the use of fungal technologies for the degradation of organic compounds has begun. Thus, the need has arisen for a book that discusses the unique role of fungi in bioremediation.

The state of the science described here represents pioneer work that focuses on the new and exciting field of mycoremediation. The book contains elements from all scientific and engineering disciplines known globally and lays a strong foundation in the subject that will serve to connect knowledge developed in both the twentieth and twenty-first centuries. The book is encyclopedic in scope and presents various types of fungi and the associated fungal processes used to clean up wastes and wastewaters in contaminated environments. The book covers aspects related to degradative fungi, taxonomy, biochemistry, enzymology, reactor engineering, genetic engineering, ecology of

biodegradation, and practical applications. The knowledge flows broadly from fundamental to practical aspects, making it useful to learn and apply bioremediation holistically. The book contains not only an interwoven synthesis and historical perspective of the technology but also provides "slow-release nutrition" for inventions and new frontiers for future research.

Fungi are one of nature's most versatile organisms in their structure, metabolism, ecology, and genomics. The first step is to understand fungal morphology, analysis and measurement of growth, and processes of fungal biodegradation (Chapter 1). Immunological and molecular assays are defined as novel tools for the detection of degradative fungi. Chapters 2 and 3 cover the treatment of a wide variety of industrial wastewaters and brewery and distillery wastes, fermentations, bioreactors, and modeling concomitant with economic importance. Next, the metabolic pathways and mechanisms of mycotransformation and mycodetoxification of petroleum hydrocarbons (Chapter 4), polychlorinated biphenyls and dioxins (Chapter 5), and pesticides (Chapter 6) are explored. A wide spectrum of bioreactors, mechanisms, and factors affecting mycotransformation, metabolic pathways, and metabolites of phenols (Chapter 7), polycyclic aromatic hydrocarbons (Chapter 8), pulp and paper mill effluents (Chapter 9), and dyes (Chapter 10) are covered next. The role of fungal enzymes in the degradation and transformation of phenols, polycyclic aromatic hydrocarbons, lignin and pulp and paper mill effluents, and dyes is discussed in detail. Chapter 11 focuses on the properties, associated mechanisms, and applications of living and nonliving fungal biomass in metal biosorption. The role of mycorrhizal fungi in the uptake of toxic metals and degradation of xenobiotic organic compounds is also discussed (Chapter 12). Methods to identify, select, and use fungi are discussed holistically associated with mycoremediation applications. The latest advances in genetic engineering and molecular biotechnologies that will be useful for the creation of suitable fungi capable of faster detoxification of these compounds are also described. However, many problems and limitations still exist and need to be overcome.

The book is intended to reach a wide audience, including managers and leaders in research and the practice of mycoremediation and should be very useful as a reference tool for practicing engineers, scientists, waste site managers, and regulatory experts. It will also provide useful information for experts in allied fields, such as botany, mycology, geology, ecology, fungal biochemistry, genetics, enzymology, metabolic engineering, environmental microbiology, and biotechnology. This should be a leading source for graduate and undergraduate students interested in understanding the capacities and processes of fungal biodegradation. Graduate students can conduct experiments or research in the laboratory or apply fungi in bioremediation at contaminated sites without seeking special guidance. The work will also serve as a handbook for the creation of new designs and components for mycoremediation processes. Instructors will find that they can teach a course or course-related topics directly from the book, and lectures may be prepared in a

variety of programs of study, from fundamental background to case studies. The nearly 2000 references can serve as a template for any specific subject area related to mycoremediation. The book will stimulate thought and greater research in the wider context of mycoremediation processes in the coming decades. Hazardous wastes and wastewaters constitute a problem of modern civilization that will not go away for centuries. New wastes and wastewaters are being generated every year with our growing industrialization. A day will come when fungi will play a greater role in the transformation and detoxification of hazardous wastes and wastewaters than at present.

I am highly grateful to Dr. Steven C. McCutcheon for his valuable guidance and suggestions during preparation of the book. Special credit goes to my wife, Dr. Julu Bhatnagar, who provided insight in biotechnology and molecular and immunological techniques for the detection of degradative fungi as well as contributing to the design of the cover. Finally, I wish to express my sincere thanks to Mr. Nathan Wilson for allowing me to use his photograph of *Trametes versicolor* (from the Web site http://mushroomobserver.org) on the cover of the book.

Disclaimer

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Atlanta, Georgia   Harbhajan Singh

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INTRODUCTION

Fungi are a diverse group of organisms and are ubiquitous in the environment. They have contributed a lot toward shaping human welfare since the beginning of civilization. Their contribution ranges from natural to industrial use. They can exist and survive in almost every habitat. Fungi play vital roles in all ecosystems and are capable of regulating the flow of nutrients and energy through their mycelial networks. They affect the environment at the macroscale, although their impact remains largely hidden from the world. Their mycelial networks may cover several hectares of forest floor, and thus fungi are considered the natural and true ecosystem engineers (Lawton and Jones, 1995). However, the contributions of the fungi are often neglected by professionals, engineers, scientists, and the general public.

Fungi are believed to have survived for about 5300 years (Haselwandter and Ebner, 1994). *Armillaria bulbosa* is considered among the largest and longest-living organisms in the world (Smith et al., 1992). Fungi are microscopic eukaryotic organisms exhibiting growth on various substrates and are capable of continuing their function almost indefinitely. These organisms, including the molds, yeasts, and filamentous fungi, are unique microorganisms that can be employed in the remediation of wastes and wastewaters. Environmental engineers all over the world have to solve problems of wastes and wastewaters, and many saprophytic filamentous fungi can degrade compounds flowing with wastewaters to receiving waters and thus contribute to their cleanup. Some molds, yeasts, and fungi are highly tolerant to extremes of hydrogen ion concentration in the substrate and can be found in a very

acidic or highly alkaline environment. Fungi are highly plastic and most fungal cells are totipotent, so the entire organism can be regenerated not only from spores but also from hyphal fragments.

1.1   FUNGAL BIODEGRADATION AND BIODETERIORATION

Fungi are known to degrade, or cause to deteriorate, a wide variety of materials and compounds, processes known as *mycodegradation* and *mycodeterioration*. The degradative activities of fungi have been recognized in various situations where they destroy different types of wood, stored paper, textiles, plastics, leather, and electroinsulating and various wrapping materials (Table 1.1). Polyethylene, with a molecular weight of 4000 to 28000, is degraded by the cultivation of *Penicillium simplicissimum* YK (Yamada-Onodera et al., 2001), and bioremediation of polyethylene may be possible in the future. Enzymes of *Mucor rouxii* NRRL 1835 and *Aspergillus flavus* have produced changes in the mechanical properties and weight of disposable polyethylene bags (El-Shafei et al., 1998). The white-rot fungi are also efficient in polyethylene degradation (Iiyoshi et al., 1997). *Aspergillus flavus* colonized and degraded chitosan-graft polymethyl methacrylate film by 45% during 25 days of aerobic cultivation in a study by Harish Prashanth et al. (2005). *Phanerochaete chrysosporium* attached to fibers of polyamide-6 and reduced 50% of the polymer's molar mass after 3 months (Klun et al., 2003). Of 15 species of white- and brown-rot fungi, *Resinicium bicolor* was shown to be the most effective fungus for the detoxification of ground waste tire rubber material prior to devulcanization (Bredberg et al., 2002).

Fungi also assist in the deterioration of concrete. Species of *Fusarium* contribute to a loss of calcium and weight in concrete during exposure (Gu et al., 1998). Increase in cement porosity and a loss of bending strength with low leaching of calcium have been attributed to acids produced by *Aspergillus niger* and *Mycelia sterilia* (Perfettini et al., 1991). Direct contact of fungal mycelia with a cement surface is not necessary. Black fungi such as *Phoma* and *Alternaria* interact with marble surfaces and cause physical, chemical, and aesthetic damage (Diakumaku et al., 1995). *Aureobasidium pullulans* colonizes on plasticized polyvinyl chloride (pPVC) within 25 to 40 weeks of exposure (Webb et al., 2000). *Aureobasidium pullulans* grows on intact pPVC formulation as a sole source of carbon, secretes extracellular

TABLE 1.1   Materials for Fungal Biodegradation and Biodeterioration

Wood	Plastics	Library materials
Wooden artifacts	Wool	Wall paintings
Stored paper	Wrapping papers	Electroinsulating materials
Textiles	Glass surfaces	Coal
Leather	Concrete	Ground waste rubber materials

esterase, and causes weight loss of the substratum. In addition, new construction materials produced from crushed plant parts are highly endangered by the molds.

Fungal biodeterioration of various types of library materials is a problem worldwide. In the period 1919–1977, filamentous fungi belonging to 234 species of 84 genera were isolated from different types of library materials (Zyska, 1997). Twenty-five years of research investigating stains on paper made by fungi, known as *foxing*, have been described (Arai, 2000). A culture of *Rhizopus oryzae* converted waste office paper to L(+)-lactic acid (Park et al., 2004). Fungi also cause decay in wall paintings and deterioration of wooden artifacts. There is a vast literature on wood decay by white- and brown-rot fungi. Fungi have also been known to attack ancient and medieval glass surfaces. *Trichophyton simii* and *A. niger* have been shown to cause degradation of wool in a liquid culture medium (Shrivastava et al., 1996). Both fungi cause a weight loss in wool and the release of proteins. Some fungal species are also known to invade resins used to glue together the lenses of microscopes and other optical instruments. *Fusarium moniliforme* is capable of degrading deoxyribonucleic acid (DNA) to nucleic bases or their derivatives (Kruszewska et al., 2004).

1.2 HOW A FUNGUS ESCAPES WATER TO GROW IN AIR

Filamentous fungi can colonize moist substrates such as wood and breach the water–air interface to grow into the air (Wosten et al., 1999). *Schizophyllum commune* reduces the surface tension of water before escaping the aqueous phase to form aerial hyphae or fruiting bodies. Surface tension results from the secretion of large amounts of hydrophobin into the aqueous surroundings that form a stable amphipathic protein film at the water–air interface. In a continuous flow of liquid medium, the hyphae of mycelial fungi, especially of *A. niger*, reorient their direction to grow upstream (Oh et al., 1997).

1.3 FUNGAL MORPHOLOGY, ANALYSIS, AND GROWTH MEASUREMENT

1.3.1 Fungal Morphology

The fungal kingdom represents a group of organisms with a great variety of structures. The majority of fungi are composed of vegetative structures termed *mycelia* that grow from their tips as binary branching trees (Viniegra-Gonzalez et al., 1993). Mycelia are unique threadlike, tubular structures that form extensive branched and anastomosing systems. Each mycelium is like a forest, where an individual tree is known as a *hypha*. The hyphae of most fungi are septate, but the presence of pores through the septa allows the mass

movement of cytoplasm and organelles and the migration of nuclei through the mycelium to distant parts. Each hypha starts from a spore that upon germination produces one or several outgrowths called *germinal tubes*, where material is transported toward the apex for growth. Hyphae are bounded by a wall of a rigid heteropolymer matrix called *chitin*, which is composed of glucosamine. A mycelium contains two types of vesicles in the cytoplasm that play an important role in apical growth (Bartnicki-Garcia, 1990). These vesicles are of different sizes, perform different functions, and are recognized as macrovesicles and microvesicles. *Macrovesicles* secrete the polymers and enzymes that comprise the amorphous phase of the wall, and *microvesicle* components constitute the chitin skeleton of the wall. Components of macrovesicles are synthesized internally, whereas the components of microvesicles accumulate at or near a wall–membrane interface. Yeasts are unicellular organisms with a cell wall and a single nucleus per cell. In general, there is considerable variation in the form of yeast cells, but there are two basic types: budding and fission yeasts.

Filamentous fungi show several distinct morphologies in submerged cultures. Mycelia are freely dispersed or become agglomerated in the medium. Loose hyphal aggregates are referred to as *clumps*; denser, often spherical aggregates are termed *pellets*. Hyphae can range from linear filaments to branched structures. Clumps and pellets vary in size, density, and surface structure, even in the same fungus. Due to their viscous nature, the dispersed mycelial suspensions behave in a non-Newtonian manner. Pellet fungal suspensions are less viscous and thus exert less influence on the flow properties of a bulk liquid medium. The rheology of fermentation fluids is affected by two primary factors: biomass concentration and fungal cell morphology. Fungal cell morphology is influenced by the agitation intensity, culture CO_2, pH, specific growth rate, and O_2.

1.3.2 Analysis of Fungal Morphology

Analysis of fungal morphology depends on accurate measurement of hyphal length and branching frequency. A variety of methods are recognized for the analysis of fungal morphology (Table 1.2). Image analysis is one of these methods. A hyphal growth unit (G) has been proposed (Caldwell and Trinci, 1973) to define the total hyphal length (main hyphae plus branches) divided

TABLE 1.2   Methods for Analysis of Fungal Morphology

Method	Reference
Autoradiography	Pitt and Bull, 1982
Image analysis	Cox et al., 1998
Automated image analysis and software	Dorge et al., 2000; Nelson et al., 2000
Automated image analysis and fluorescent staining	Morgan et al., 1991

by the number of tips, the sites of hyphal extension. Autoradiographic studies have shown the nonviability of the significant percentage of hyphal tips in *Trichoderma* that does not contribute to growth (Pitt and Bull, 1982). The value of G accounts for the number of viable tips. G is calculated on a volume basis (G_{vol}) if variations in individual hyphal diameters are also noted (Robinson and Smith, 1979). Estimation of a precise G_{vol} value requires the use of two magnifications to measure hyphal width (Cox et al., 1998).

An automated image analysis program and protocol have been developed for the identification and enumeration of live versus dead cells of *Aureobasidium pullulans* on microscopic slides and leaf surfaces (Nelson et al., 2000). Live cells take up CellTracker Blue and nonviable DEAD Red, and the Optimas software is used to differentiate viable and dead cells. An automatic method of creating digital images of fungi was employed to evaluate the color distribution, colony dimensions, and texture measurements of seven isolates of *Penicillium* (Dorge et al., 2000). A Windows application could locate the position and size of up to three colonies in an image. Automated image analysis and fluorescent staining have also been used to determine the fungal biomass in soil and on inert surfaces (Morgan et al., 1991).

Several measurements are used for loose aggregated mycelia (clumps) in a dispersed state. In common use is projected area measurement, in which a three-dimensional area of a clump can be captured to a reasonable size. In *Aspergillus niger*, ratios of the clump perimeter to the core perimeter have been used to measure clump shape (Papagianni et al., 1999). The projected clump area correlates well with broth rheology for *Penicillium chrysogenum* (Cox et al., 1998). Projected area measurements are also employed for the image analysis of fungal pellets, pellet size, and the shape and dimensions of a protruding hyphal region. However, a measurement related to all morphological forms in fungal fermentations has yet to be developed. In filamentous fungi, fractal analysis has been used with some success to study colony growth (Jones et al., 1993; Matsuura and Miyazima, 1993). A standard method to measure fractal dimensions (D) is unknown, and the D value obtained is method dependent (Soddell and Seviour, 1994).

1.3.3   Pellet Formation and Structure

The processes in the formation of pellets in fungi are not fully understood, but two different mechanisms, coagulating and noncoagulating pelletization, have been proposed. The formation of coagulating pellets is a two-step process: the aggregation of spores, and subsequent aggregation of germinating spore agglomerates. Noncoagulating pellets result from germination of a single spore. *Phanerochaete chrysosporium* appears to involve surface polysaccharide bridging during the initial step in aggregation, but it is unclear whether this mechanism is applicable to all fungi (Gerin et al., 1993). Pellets may also result from the agglomeration of hyphal aggregates and fragments of previously broken pellets. Factors affecting the formation of pellets are

strain specific, and the pellet structure usually depends on the cultivation conditions used during the process. The structure of mycelial pellets is variable and can range from loose, irregular aggregates to tight, compact spheres. In general, pelleted suspensions of fungal cells are not viscous and deviate from Newtonian behavior at high biomass concentrations.

Several factors are recognized to affect the formation and structure of pellets in filamentous fungi (Gibbs et al., 2000): inoculum concentration and preparation, agitation intensity, composition of the medium, and others. Medium composition includes the culture pH, carbon level, phosphorus concentration, surface-active agent, and so on. Some fungi do not form pellets; other fungi in pelleted form exhibit reduction in the production of metabolites caused by nutrient transfer limitations in the cells of pellets. Tightly compact pellets are deficient in the free flow of molecular diffusion and are hollow in the center, due to autolysis of the mycelium. In *Penicillium chrysogenum*, pellets with a diameter of less than $400\mu M$ contain all active cells. Larger pellets exhibit four distinct zones: an outer layer of actively growing cells, two layers of less active cells, and a hollow center. Only the outer 100 to $200\mu M$ layer of *Phanerochaete chrysosporium* pellets assimilates glucose (Cronenberg et al., 1994). A possible relationship exists between fungal morphology and metabolite production in filamentous fungi.

1.3.4 Growth Measurement

Growth is a complex process with many different component processes. Regulation of growth is controlled by numerous factors. Fungi require carbon and nitrogen for growth, and some aspects of nutrition have been examined by Singh (1978). Various media have been established for the detection and enumeration of yeasts and molds (Beuchat, 1992). The spread-plate technique is preferred over the pour-plate technique. Methods exist for the measurement of growth in fungi (Table 1.3). Chitin measurement is used to estimate fungal biomass in solid-state fermentation (Roche et al., 1993). Ergosterol is also considered to be an indicator of fungal biomass. As ergosterol is subject to significant photochemical degradation, this test should be employed carefully as a biomarker for living fungi (Mille-Lindblom et al., 2004). A simple analytical technique has been developed that enables quantitative determination of fungal growth and decolorizing ability in solid media for white-rot fungi

TABLE 1.3   Growth Measurement of Fungi

Method	Reference
Chitin measurement	Roche et al., 1993
Microtiter plate and microplate reader	Langvad, 1999
Colony-forming units	Bridge and Spooner, 2001
Electrochemical	Subrahmanyam et al., 2000
Voltammetric	Ci et al., 1997

(Yonni et al., 2004). This technique is used on various solid media containing a low or high nitrogen content and on dyes.

In general, fungal growth in liquid culture is measured in terms of dry weight in either stationary or shake cultures in 500-ml Erlenmeyer flasks. A rapid and efficient method has been developed for the measurement of growth in filamentous fungi (Langvad, 1999). Growth can be measured very efficiently on a 96-well microtiter plate and microplate reader. Enumeration of fungal propagules by colony-forming units (CFU) is employed routinely for fungal quantification (Bridge and Spooner, 2001).

An electrochemical method based on cyclic voltammetry shows all four phases of *Fusarium solani* growth (Subrahmanyam et al., 2000). The growth curve obtained matches well with conventional methods of measuring dry weight. The experiments also confirm that the anodic peak is due to metabolites, not the fungal biomass. The behavior of living cells of *Saccharomyces cerevisiae* in a voltammetric system with a graphite electrode indicates the relationship between the peak current and growth curve during the entire period of fermentation (Ci et al., 1997). The early stages of growth, from germination through hyphal development of filamentous fungi such as *Penicillium*, *Phoma*, and *Trichoderma*, can be analyzed by flow cytometry (Bradner and Nevalainen, 2003).

1.4   MASS TRANSFER, GROWTH KINETICS, AND BIOREACTORS

Filamentous fungi are the most important microorganisms for the processing of solid substrates. This can be attributed as due to the diverse capabilities and hyphal mode of growth. Finite difference was the first model to predict changes in hyphal length, branching, and septa, based on the cellular mechanisms observed (Prosser and Trinci, 1979). Since then, several models have been proposed to elucidate hyphal growth by apical enlargement and branching. These models have been divided into two categories and described by Nielsen (1992). In general, cell models are based on the cellular mechanisms observed, and population models consider a number of intracellular components and reactions. A range of modeling approaches is applied to vegetative growth in fungi. These include empirical, mechanistic, deterministic, and stochastic models.

Bartnicki-Garcia et al. (1989) proposed an empirical model to describe apical growth and hyphal morphogenesis qualitatively. Two main parameters are used: the rate of increase in area equal to the number of vesicles released by the vesicle-supplied center (VSC) per unit time, and the rate of linear displacement of the VSC. Georgiou and Shuler (1986) considered diffusion of the substrate and biomass in the colony in terms of vegetative, competent, conidiophore, and conidial species. Model simulation suggested the proliferation of differentiating structures due to mass transfer limitations. Aynsley

et al. (1990) developed a kinetic model for the growth of mycelial fungi in a submerged culture. Nutrients are absorbed uniformly, transformed into wall precursors, and transported to the growth tip, where they are utilized in the process of extension. No mass transfer limitation is assumed here and the precursor is transformed at a constant rate via Michaelis–Menten kinetics. Mitchell et al. (1991) proposed a semimechanistic model for the growth of *Rhizopus oligosporus*, secreting glucoamylase on a solid medium where it diffuses during the hydrolysis of starch and the release of glucose. Glucose absorbed by the mycelium is transformed into biomass via Monod kinetics. A mathematical model has been developed for apical growth, septation, and branching of mycelial fungi (Yang et al., 1992). The model is based on the diffusion and production of wall precursors to describe tip growth and septation (deterministic) and for the branching (stochastic) processes. Morphological development of mycelia up to the formation of pellets correlates well with experimental findings.

A mechanistic model has been derived that considers mass transfer and hyphal growth in filamentous fungi before branching (Lopez-Isunza et al., 1997). The model considers the substrate diffusion in a solid medium, uptake, and release of substrate at the wall by a carrier, and formation of wall precursors to mass transport by diffusion and convection inside the hyphae. Good qualitative correlations have been shown between model simulations for a range of substrate concentration with the experimental data when measured values of hyphal diameter and the maximum germ tube length are considered. Cui et al. (1998) presented a mathematical model of fungal growth and morphology in submerged fermentations. This model accounts for oxygen transfer, agitation intensity, dissolved oxygen tension, pellet size formation of mycelia, the fraction of mycelia in the total biomass, carbohydrate source consumption, and biomass growth. Comparison of simulation with experimental data indicates that the model can describe well the period of fungal growth and morphology. Variations in hyphal tensile strength with time have been considered that can lead to improvement in the morphology models (Li et al., 2002). A mathematical model has been developed that relates the properties of a fungal mycelium at the microscopic level to growth and function at the macroscale (Boswell et al., 2003). The model is based on movement of the hyphal tips and the translocation of internal substrate. The model equations are of mixed hyperbolic–parabolic type and it is important that the mass be conserved and maintained. The model is calibrated for *Fusarium solani* growing on a glucose–mineral salts medium and solved in both homogeneous and heterogeneous environments.

A model has been constructed to explain the colony patterning and collective hyphal growth of filamentous fungi (Matsuura, 2002). The model considers two external parameters, such as the initial nutrient level and the step length of a nutrient random walk, and one internal parameter, such as the frequency of nutrient uptake. The model colony shows the onset of disordered ramifications at low nutrient levels, with reduced nutrient uptake

frequency under diffuse conditions. During the growth of *Aspergillus nidulans*, the rate of hyphal extension is reduced, due to a population effect when hyphae produce three-dimensional dense colonies. The colony periphery becomes thick, due to a population effect as the nutrient diffusion effect is increased at high nutrient levels with a low rate of hyphal growth. The pattern of colony and onset of disorder are propelled by a combination of nutrient diffusion and rate of hyphal growth.

Certain fungal bioreactors have been used for the removal of several pollutants (Table 1.4). Fungal bioreactors are also termed *mycoreactors*. Mycoreactors are of two types: (1) biofilm or immobilized systems and (2) suspended growth. Mycoreactors based on attached growth include trickling filters, rotating biological contactors, upflow fixed-film reactors, and fluidized-bed reactors, and those based on suspended growth include slurry reactors, among others. Different mycoreactors are operated under aerobic or anaerobic conditions. In mycoreactors, the operation can be batch, semibatch, sequencing batch, or continuous. Stirred tanks are the most common mycoreactors for submerged growth. However, under certain conditions, other reactor types, such as packed bed, bubble column, and airlift tower loops, are also used. Several fungi are used in immobilized form in various reactors to facilitate rapid biodegradation. Pellet-forming fungi are cultivated in aerated fluidized-bed or suspended airlift loop reactors. Several physical, chemical, and biological parameters affecting the bioprocess are important in the monitoring and control of performance and of bioreactors and in their improvement. A mathematical process modeling involving various assumptions can be used to predict the efficiency of contaminant removal from bioreactors. The metabolic and genetic diversity of fungi in bioreactors can be analyzed by obtaining polymerase chain reaction (PCR) amplification of DNA. The use of ultrasonic field-based filters has been assessed for the enhanced separation of filamentous fungi and its use in repeated-batch processes (Crognale et al., 2002). The operating conditions were optimized, and three batch processes revealed 88% of a system's separation efficiency.

During the past decade, knowledge-based control systems have been used in biotechnological processes. The knowledge-based part of the Biogenes control system for bioprocess control of fed-batch *Saccharomyces cerevisiae* cultivation has been outlined (Hrncirip et al., 2002). The process data

TABLE 1.4   Typical Fungal Bioreactors (Mycoreactors) for Removal of Pollutants

Trickling filter	Packed bed
Rotating biological contactor	Bubble column
Upflow fixed film	Fluidized bed
Slurry	Airlift tower loop
Stirred tank	Immobilized (various types)

classification, qualitative process state identification, procedures of online identification of metabolic states, and supervisory process control and performance of the system from several cultivations were discussed.

1.5 METHODS FOR DETECTION OF DEGRADATIVE FUNGI

Fungi are important in the functioning of a soil ecosystem, especially the decomposition processes of organic matter. Unfortunately, little is known as to the population structure, dynamics, and diversity of fungi in soil. Some methods are designed to detect viable decay fungi, and others detect residual nonviable fungal metabolites. Direct cultivation methods are employed for assessment of the prevalence of microorganisms in soil, but fungal growth on agar plates may not present an accurate picture of the in situ structure and diversity of fungal communities. Moreover, mycorrhizal fungi are reluctant to grow on agar plates. It is also difficult to make inferences about in situ fungal numbers based on observations from plates. Thus, it is necessary to develop novel methods to study the in situ behavior of fungi in processes related to ecology, decomposition, and bioremediation. Methods selected for the detection of degradative fungi have been described by Jellison and Jasalavich (2000).

1.5.1 Immunological Assays

Immunological assays have been used for the detection of fungi in culture and the detection of decay fungi in wood since the mid-1980s (Table 1.5). Monoclonal antibodies to *Postia placenta* using extracellular culture filtrates were first produced in 1986 (Jellison and Goodell, 1986). Monoclonal antibodies to detect fungal metabolites such as manganese peroxidase (MnP) in wood by *Phanerochaete chrysosporium* (Daniel et al., 1991) and polyclonal antibodies for laccase by *Lentinus* (*Lentinula*) *edodes* (Goodell et al., 1998) were subsequently recognized. Clausen (1997) reviewed the diversity of immunological techniques from 1986 to the present and their application to wood decay fungi. These techniques include immunological probes, immunofluorescence, immunoblotting, agglutination assay, enzyme-linked immunosorbent assay (ELISA), modified ELISA, dipstick assay, and chromatographic assay.

TABLE 1.5 Immunological Assays for Detection of Degradative Fungi

Monoclonal antibodies	ELISA
Polyclonal antibodies	Modified ELISA
Immunofluorescence	Chromatographic assay
Immunoblotting	Agglutination assay
Dot immunoblotting	Dipstick assay

A list of various preparations was assessed for the production of polyclonal antibodies against a number of brown-rot and stain fungi using immunological probes (Clausen, 1997). Monoclonal antibodies to both whole-cell mycelial extracts and extracellular antigen extracts of *Serpula lacrymans* have been produced (Glancy and Palfreyman, 1993). Immunofluorescence has been studied as a sensitive and quantitative analysis for the detection of Basidiomycetes in wood. Dot-immunoblot assay on nitrocellulose paper with polyclonal antibodies to the brown-rot fungus *Lentinus* (*Lentinula*) *lepideus* was first reported by Glancy et al. (1989), and this assay can be a preliminary test for detecting brown-rot decay (Clausen et al., 1990). Western blot is used to differentiate isolates of *Serpula lacrymans* from other wood decay basidiomycete fungi (Viagrow et al., 1991). Agglutination assay, considered to be the most rapid immunodiagnostic technique, is a good field test for brown-rot decay (Clausen et al., 1991). ELISA assays have been employed for the quantitative detection of *Ophiostoma* sp. C28 within wood using polyclonal antibody (Breuil et al., 1990). Modified ELISA has also been utilized on wood for the detection of *Ophiostoma* sp. C28. Strong ELISA reactions have been obtained in copper chromated arsenate– and ammoniac copper–treated wood infected by *Phialophora mutabilis* using polyclonal antibodies (Daniel and Nilsson, 1990). A dipstick immunoassay has been developed for the detection of *Phytophthora cinnamomi* in soils (Cahill and Hardham, 1994). An indirect immunofluorescence test has been developed to detect 10 propagules/ml of *Hormoconis resinae* in aviation kerosine in 5 hours (Lopes and Gaylarde, 1996). ELISA has also been discussed for the quantitative estimation of fungal biomass during solid-state fermentation (Dubey et al., 1998).

1.5.2   Molecular Assays

Recently, molecular strategies for the detection of fate and diversity of fungi in soils have been developed. These methods can identify fungi on a species level and are based on the use of variable internally transcribed spacer regions. A PCR has been used to amplify rDNA sequences selectively to determine the total fungal diversity and taxonomy using universal primers (White et al., 1990). Specific primers can be designed to amplify and analyze fungal DNA from cultures. Table 1.6 shows available molecular assays for the detection of degradative fungi in various media.

TABLE 1.6   Molecular Assays for Detection of Degradative Fungi

Polymerase chain reaction (PCR)
PCR followed by denaturing gradient gel electrophoresis or agarose gel
  electrophoresis or microchip electrophoresis
Restriction fragment length polymorphism analysis of PCR
Real-time PCR (TaqMan OCR and ABI Prism Sequence Detection)
In situ hybridization

The 18S ribosomal RNA sequence as a phylogenetic marker to distinguish different fungal species is less developed than the 16S rRNA sequence in bacteria. About 114 rRNA sequences of four major divisions of fungi were known up to 1995 (van de Peer and De Wachter, 1995), but this number has now increased to 500. Fungal-specific primer pairs EF4f/EF3r and EF4f/Fung5r can be used to amplify a wide range of fungi of the major groups (Smit et al., 1999). Profiles of fungal communities in soil have been assessed via fungal-specific PCR, followed by denaturing gradient gel electrophoresis (van Elsas et al., 2000). Of four primer pairs, the primer pair EF4f/Fung5r produced amplicons with the DNA of a wide range of fungi. The phylogenetic position of a variety of fungal species has been investigated. Fungal diversity from microbial communities can be assessed by internal transcribed spacer (ITS) rDNA terminal restriction fragment (TRF) pattern analysis (Lord et al., 2002). Borneman and Hartin (2000) reported on two newly developed primer pairs to amplify rDNA from all major classes of fungi in soil. Detection and quantification of fungal DNA from soil substrate involve specific primers targeting a 362-base pair (bp) fragment from the SSU rRNA gene region of *Glomus intraradices* and a 562-bp fragment from the *Fusarium solani* f.sp. *phaseoli* translation elongation factor 1 alpha gene in real-time PCR conjugated with the fluorescent SYBR Green I dye (Filion et al., 2003). New specific primers AR1 and AR2 are used to amplify a specific part of the ITS region of rDNA of *Armillaria* isolated from soil samples (Lochman et al., 2004). The ITS region is amplified by nested PCR with external primers ITS1 and ITS4 and internal primers AR1 and AR2. Species of *Armillaria* are differentiated by analysis of restriction fragment length polymorphisms (RFLPs) with the restriction endonuclease HinfI.

A protocol has been developed for the direct extraction of fungal DNA from soil (Nazar et al., 1996). The fungal DNA extraction method (FDM), modified from the protocol of Nazar et al. (1996) and proposed by van Elsas et al. (2000), is efficient in lysing fungal spores or mycelia. In addition, several methods are known to prepare amplifiable DNA for PCR from fungal conidia. Three methods for DNA extraction from fungal conidia and yeast cells in air, water, and dust samples have been evaluated for use in real-time PCR (TaqMan) analyses (Haugland et al., 2002). A simple bead milling method has been developed for accurate estimation of these organisms in air and water (tap and surface) samples. A total of 120 fungal strains were tested by multiplex PCR followed by agarose gel electrophoresis (PCR-AGE) or microchip electrophoresis (PCR-ME) (Fujita et al., 2001). The internal transcribed spacer 1 (ITS1) and ITS2 regions and 5.8S rDNA region were amplified using universal primers ITS1 and ITS4. The ITS2 region was amplified simultaneously using universal primers ITS3 and ITS4. Isolates of the dry-rot fungus *Serpula lacrymans* were detected by the PCR of the internal transcribed spacer region (ITS1, 5.8S rDNA, ITS2) using universal primers ITS1 and ITS4 coupled with restriction digestion of the resulting product to produce restriction fragment length polymorphisms (RFLPs) (Schmidt and Moreth,

1998). RFLP analysis of PCR-amplified rDNA has also been used to characterize tropical wood-decaying fungi (Zaremski et al., 1998). Jasalavich et al. (2000) amplified the internal transcribed spacer regions of both Ascomycetes and Basidiomycetes from pure culture and inoculated spruce wood using universal primer ITS4 and primer pair ITS1-F, specific for higher fungi. TaqI RFLP profiles clearly separate several species of Basidiomycetes and subspecies of *Phanerochaete chrysosporium* and *Scytinostroma galactinum*. Isolates of *Trametes versicolor* can be distinguished at the species level by a AluI RFLP profile and TaqI RFLP profiles and at the subspecies level by HaeIII RFLP profiles. The basidiomycete-specific primer system in conjunction with RFLP analysis of the amplified product is both selective and specific in detecting white- and brown-rot fungi and is a potential tool for the study of fungal colonization and wood degradation.

In situ hybridization can assist in the rapid and accurate identification of filamentous fungi in histologic tissue sections (Hayden et al., 2002). In this study, DNA probes are directed against the 5S or 18S ribosomal RNA sequences of these fungi. Rapid and efficient extraction of fungal DNA from small quantities of tissue in a batch-processing format has been examined (Griffin et al., 2002). This assay involves scraping of tissue, a boiling step, and use of the Qiagen DNeasy Plant Tissue Kit protocol to purify the DNA for PCR/sequencing. Ultraviolet (UV) matrix-assisted laser desorption/ionization (MALDI) time-of-fight (TOF) mass spectrometry (MS) has been used for the rapid identification and characterization of *Saccharomyces cerevisiae* (Amiri-Eliasi and Fenselau, 2001).

1.6   FUNGI AS ENVIRONMENTAL INDICATORS

In the past decade, the use of fungi as bioindicators in air, soil, and water pollution surveys has been recognized. However, fungal species are unable to map pollution. Of four fungi, *Thelephora caryophyllea* accumulates metals in the soil (Maurice and Lagerkvist, 2000). It is possible to use an arsenic test with fungi from a preliminary assessment point of view. *Lycoperdon perlatum* may be employed as a bioindicator of heavy metals and selenium in soil pollution (Quinche, 1990). The mycological quality of the sand beaches of Lisboa and Vale do Tejo, Portugal, indicates that *Scopulariopsis* and *Candida* can be used as specific environmental indicator organisms of sand beach quality (Mendes et al., 1998). ^{137}Cs/^{134}Cs activities have been measured in about 250 soil samples and 350 samples of fungal species in a forest in Bavaria, Germany, and this ratio has been found to be an indicator of major mycelium locations in forest soil (Ruhm et al., 1997). Two approaches, static and dynamic, have advantages in estimating the mycelium location of fungi.

Yeasts are used in various tests for the determination of mutagenic or carcinogenic action. Due to limited permeability, yeast cells exhibit lower sensitivity to mutagens or carcinogens than do bacteria. The general perme-

ability of *Saccharomyces cerevisiae* cells can be enhanced by mutation, and on this basis, a more sensitive test (D7ts1) has been developed to study environmental pollution (Terziyska et al., 2000). This test measures positive samples of soil, air, and water and detects mutagenic or carcinogenic activities. The new D7ts1 test has proved better than the yeast D7 test of Zimmerman. All samples with a positive bacterial Ames test are positive in the new D7ts1 test. However, some samples positive in D7ts1 tests are negative in the Ames test. Simultaneous use of the D7ts1 and Ames tests is beneficial, as it detects dangers for activities related to human health where bacterial cells do not respond.

Recent advances in knowledge of multicolored fluorescent proteins from yeasts and fungi have opened a door regarding the sensing systems used for environmental pollutants. Whole cells of these organisms can be incorporated into different array formats on silicon chips, optic fibers, and other configurations. Baronian (2004) discussed the use of the whole cells of yeast and filamentous fungi as sensing elements in biosensors.

1.7   FUNGAL ATTACK ON COAL

The role of fungi in the transformation of coal has been recognized for a decade or so. Of more than 750 fungal strains, only six strains have been shown to modify the physicochemical properties of pieces of hard coal (Hofrichter, 1998). The attack by *Coprinus sclerotigenis* C-142-1 of powdered hard coal releases 2-hydroxybiphenyl, alkylated benzenes, and polycyclic aromatic hydrocarbons. *Fusarium oxysporum* and *Trichoderma atroviride* are able to solubilize low-rank coal (LRC) (Monkemann et al., 1997). Strong evidence exists for the existence of components of a ligninolytic system comparable to *Phanerochaete chrysosporium*. *T. atroviride* secretes a partly inducible heat-sensitive agent that has hydrolytic properties and assists in the solubilization of Rhenish lignite (Holker et al., 1999a). This mold also appears to produce an unusual esterase for the cleavage of ester bonds in the presence of lignite (Holker et al., 1999b). Moreover, *T. atroviride* produces alkaline substances and chelators that assist in the dissolution of coal humic materials. *Trichoderma* sp. M2 and *Penicillium* sp. are new molds that tend to modify and solubilize hard coal without the formation of tarlike products (Laborda et al., 1997, 1999). An indigenous culture of *Aspergillus* sp. eliminated 70 to 80% of total sulfur from high-sulfur coal from the coal fields of Assam in India (Acharya et al., 2005).

Bioconversion of brown coal occurs by a process of solubilization which is typical for molds, yeasts, actinomycetes, and pseudomonad bacteria, and depolymerization is limited to ligninolytic Basidiomycetes (wood-decaying and litter-decomposing fungi). Hofrichter and Fakoussa (2001) listed various groups of fungi that show solubilizing and depolymerizing activities toward brown coal or utilize it as a growth substrate. Wood-rotting fungi and their

oxidases are found to solubilize, polymerize, depolymerize, and decolorize macromolecules derived from low-rank coal (LRC) (Ralph and Catcheside, 1997). *Phanerochaete chrysosporium* depolymerizes, and decolorizes alkali-soluble acid-precipitate LRC macromolecules (AS coal) to a form unrecoverable by alkali washing. Nitrogen-limited media with hyperbaric oxygen enhance the transformation of AS coal. This appears to be due in part to oxidation by manganese peroxidase (MnP) and lignin peroxidase (LiP); however, their precise role is yet unclear.

Ligninolytic Basidiomycetes produces three enzymes—lignin peroxidase, manganese peroxidase, and laccase—that appear to be involved in the depolymerization of coal. The basidiomycete strain RBS 1k produces MnP, and the strain RBS 1b secretes oxidative enzymes with laccase and peroxidase activity before the onset of coal solubilization (Willmann and Fakoussa, 1997). The cell-free peroxidase of RBS 1k or laccase of RBS 1b in vitro is unable to promote water solubility of low-rank coals. MnP from *Clitocybula dusenii* catalyzes and optimizes the depolymerization of coal humic substances (Ziegenhagen and Hofrichter, 1998). The South American white-rot fungus *Nematoloma frowardii* b19 and four other fungi have been shown to depolymerize low-rank-coal–derived humic acids, releasing fulvic acid–like compounds (Hofrichter and Fritsche, 1997). *Nematoloma frowardii* and other fungi produce high levels of laccase mRNA in the presence of coal humic substances in the medium (Scheel et al., 1999a, b).

1.8   THERMOPHILIC, ALPINE, AND LICHEN-FORMING FUNGI

The majority of fungi are mesophilic and grow in the temperature range 5 to 35ºC, with optimum growth between 25 and 35ºC. Some fungi are psychrophilic and are capable of growth near or below 0ºC. Certain psychrotolerant fungi can survive at the very low temperature of −40ºC. True psychrophiles include some species of *Mucor* and yeasts. Thirty-two cold-adapted, psychrophilic, cold-tolerant yeast strains from alpine habitats have been characterized related to taxonomy, growth temperature profiles, and their potential to degrade phenol and phenol-related compounds (Bergauer et al., 2005). Thermophilic fungi can grow above 40ºC and are widespread in terrestrial habitats. Thermophilic fungi can be cultivated in minimal media with growth yields and metabolic rates comparable to those of mesophilic fungi. It appears that these organisms can grow in heavy metal concentrations, variable pH, or extremes of environmental conditions. Heat-tolerant fungi are known for their ability to decompose plant polymers and their potential in composting. These fungi are useful in bioconversions, where they can reduce the requirement for cooling during growth. Some extracellular enzymes are produced by thermophilic fungi and are of good scientific and commercial interest (Maheshwari et al., 2000). Unlike white-rot fungi, enzymes of thermophilic fungi are to be discovered that can be used in mycoremediation.

The occurrence of heat-resistant fungi has been revealed in soil samples from the Slovak Republic (Jesenska et al., 1992). Several heat-resistant species of fungi have been isolated and identified. Xerotolerant fungi can grow in dry environments at very low water activities. Osmotolerant fungi are found to grow in the environments at very low osmotic potential. About 20% of all known fungal species are obligate symbionts in lichens, but lichen-forming fungi remain neglected by mycologists and engineers and overlooked by industry (Crittenden and Porter, 1991). The role of thermophilic, alpine, and lichen-forming fungi in waste and wastewater treatment is largely unexplored.

1.9   MYCOREMEDIATION: FUNGAL BIOREMEDIATION

Fungi have been harnessed by humans in many diverse applications for thousands of years. In any ecosystem, fungi are among the major decomposers of plant polymers such as cellulose, hemicellulose, and lignin. Fungi have the ability to mineralize, release, and store various elements and ions and accumulate toxic materials. They can facilitate energy exchange between the aboveground and belowground systems. Fungi have proven to modify soil permeability and soil ion exchange and to detoxify contaminated soil. Edible and/or medicinal fungi also play a role as natural environmental remediators (Pletsch et al., 1999), as do aquatic fungi (Hasija, 1994). Fungi are usually slow in growth and often require substrates for cometabolism. The mycelial growth habit of these organisms is responsible for the rapid colonization of substrates. Fungal liquid cultures constitute appropriate model systems to explore the biotransformation of a wide variety of compounds. The process of fungal biotransformation of compounds, wastes, or wastewaters is termed *mycotransformation*.

Fungal treatment of wastes in nature has been known for centuries. The ubiquitous presence of fungi has allowed acclimation of some types of wastes, if not most. Most of our knowledge related to interactions between fungi and wastes is based on studies performed in the laboratory. However, during the last decade, fungi have been used in the treatment of a wide variety of wastes and wastewaters, and the role of fungi in the bioremediation of various hazardous and toxic compounds in soils and sediments has been established. Fungi have also demonstrated the removal of metals and the degradation and mineralization of phenols and chlorinated phenolic compounds, petroleum hydrocarbons, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, chlorinated insecticides and pesticides, dyes, biopolymers, and other substances in various matrices. The role of yeasts and fungi in the treatment of various wastes and wastewaters has been discussed by Singh (1991).

1.9.1   White-Rot Fungi in Bioremediation

White-rot fungi for lignin degradation have been examined for more than half a century. After the discovery of the extracellular oxidative ligninolytic

enzymes of the white-rot fungus *Phanerochaete chrysosporium*, Bumpus et al. (1985) proposed the use of this fungus for bioremediation. Enzymes involved in the degradation of wood are also responsible for the degradation of a wide variety of persistent organic pollutants. The white-rot fungus *P. chrysosporium* has emerged as an archetypal model system for fungal bioremediation. *P. chrysosporium* has the ability to degrade toxic or insoluble compounds more efficiently than other fungi or microorganisms. The numerous oxidative and reductive mechanisms of degradation make its application attractive in different matrices. There has been a plethora of review articles on *P. chrysosporium* with respect to mechanisms of degradation of recalcitrant compounds and xenobiotics. In addition to *P. chrysosporium*, several other white-rot fungi (e.g., *Pleurotus ostreatus*, *Trametes versicolor*, *Bjerkandera adusta*, *Lentinula edodes*, *Irpex lacteus*) are known to degrade these compounds. Based on the literature of the past two decades, it appears that the white-rot fungi account for at least 30% of the total research on fungi use in bioremediation. White-rot fungi have added a new dimension to the already complex system of fungal bioremediation.

During the 1980s, marketing attempts by unskilled persons resulted in failures in white-rot fungal technology. Successful use depends on a comprehensive knowledge of fungal physiology, biochemistry, enzymology, ecology, genetics, molecular biology, engineering, and several related disciplines. The field conditions and factors that induce fungal biodegradation are taken into consideration before development of the final design. Lamar and White (2001) advocated the use of four phases in their approach: bench-scale treatability studies, on-site pilot testing, production of inoculum, and full-scale treatment. A variety of substrates, such as wood chips, wheat straw, peat, corncobs, sawdust, a nutrient-fortified mixture of grain and sawdust, bark, rice, annual plant stems and wood, fish oil, alfalfa, spent mushroom compost, sugarcane bagasse, coffee pulp, sugar beet pulp, okra, canola meal, cyclodextrins, and surfactants, can be employed in inoculum production off-site or on-site or mixed with contaminated soils to enhance degradation. Care is required to balance the carbon and nitrogen ratio in the substrates, which have a significant influence on the degradative performance of white-rot fungi. Pelleted fungal inocula coated with alginate, gelatin, agarose, carrageenan, chitosan, and so on, are used by several researchers and offer several advantages over inocula produced using bulk substrates. This strategy, adapted from the mushroom spawn industry, is known as *encapsulation*. Encapsulation sustains the viability of the inoculum and provides sources of nutrition for the maximum degradation of pollutants (Bennett et al., 2001). This also enhances the survival and effectiveness of the introduced species. Solid-state fermentation (SFF) is another method for producing fungal inoculum. However, fungi secrete several by-products during conversion of agricultural waste under SFF conditions (Cohen and Hadar, 2001).

Three phases of strategies are envisioned for the successful implementation of mycoremediation. Inoculum preparation techniques and their improvements lead to success in the first phase of the use of white-rot fungi in

mycoremediation. The second phase includes clear technical protocols for the final design and associated engineering processes. The remediation protocols for the monitoring, adjustment, continuity, and maintenance of the engineering system dictate the success of the third and final phase of the mycoremediation process. Competition from native microbial populations contributes to the outcome of mycoremediation, but protocols to eliminate such variability have yet to be developed. Processes using white-rot fungi have been patented. A few companies, including EarthFax Development Corporation in Utah and Gebruder Huber Bodenrecycling in Germany, employ these fungi for soil bioremediation, but a broader use is not known at the present time.

1.10   ECOLOGY OF MYCOREMEDIATION

The literature on fungal ecology is voluminous, but little is known of the ecology and ecophysiology of white-rot or other fungi in mycoremediation. Thus, it is very important to understand the behavior and interactions of fungi in diverse ecosystems related to biodegradation of various hazardous and toxic compounds. Despite an estimate of 1.5 million species of fungi and the identification of 69 000 fungal species worldwide (Hawksworth, 1991), the limited ecology of mycoremediation is known only in fewer than 10 species of fungi. Different techniques to study such ecology are required, as these are variable from one group or class or genus or species of fungi to another.

Soil or sediment is a poor matrix for the growth of fungi, due to low levels of substrates. The soil environment is also antagonistic to mycelial growth. In general, the diversity of fungal species and the number of propagules are reduced with increasing depth, and the fungi follow the distribution of organic matter in soil horizons (Dix and Webster, 1995). That is why mycelial networks are present in each soil horizon and fluctuate with seasonal growth patterns, rate of decomposition, and time. The development of fungal communities depends on a variety of environmental and biotic factors, such as the nature of carbon materials, quality and the quantity of nitrogen, presence of specific stimulators, temperature, hydrogen ion concentration, aeration, water availability and water potential, and interspecific competition. Fungi producing small amounts of biostatic or biocidal compounds can be employed in the process of mycoremediation. Many litter-decomposing and wood-decaying Basidiomycetes grow over the mycelia of other fungi and capture their nutrients. A plethora of literature exists on the ecology of lignocellulose degradation and litter decomposition by various wood-rotting fungi. A variety of ecosystem processes and ecosystem services are provided by fungi (Dighton, 2003).

Many members of the Mucorales, including the genera *Rhizopus* and *Mucor*, are abundant in soils. These genera grow and colonize quickly and depend on soluble carbohydrates, but the enzyme activities of most Mucorales are limited. Some stress-tolerant species of *Mucor*, *Fusarium*, *Gliocladium*,

Penicillium, and *Trichoderma* grow under conditions of water and nutrient limitations, high temperature, and in the absence of oxygen and can be a target of mycoremediation.

Aquatic fungi are distributed in various groups of fungi, especially in Mastigomycetes, some in Zygomycetes, Ascomycetes, Deuteromycetes, and yeasts, and a few in Basidiomycetes. Some of these fungi remain in water, others are amphibious, and still others are aquatic for a short period of time. Aquatic fungi are found in freshwater or marine environments. The Order Halosphaeriales of Ascomycetes contains several genera and species inhabiting the marine ecosystem. Species of aquatic Hyphomycetes are well represented on almost all substrates in freshwater systems (Barlocher, 1992). Several reviews on the ecology of aquatic fungi may be found in the literature. Knowledge of the nutritional profiles and enzyme activities of aquatic fungi is limited. However, it is necessary that attempts be made to employ aquatic fungi in the mycoremediation of contaminated surface waters, groundwaters, and wastewaters.

Our knowledge of the methodologies and ecological tools used to sustain fungal biomass, activity, and enzyme production in contaminated matrices is very limited and is a great hinderance to the process of mycoremediation. Ecological modeling related to these processes is highly complex and is not known at the present time. Eco-molecular markers, microanalysis methods, and so on, can be better tools than the results of experiments. Good emphasis is essential in this direction to achieve success in the process of mycoremediation.

1.11 GENETIC ENGINEERING OF MYCOREMEDIATION

Recent advances in molecular biology, biotechnology, and enzymology are the driving force toward engineer-improved fungi and enzymes for mycoremediation. A number of the genetic engineering approaches that have been developed have proven beneficial in adding the desired qualities in metabolic pathways or enzymes. Strain manipulation is becoming easier with the exponential expansion of molecular tool boxes and genome sequences. However, the best source is that of the genes of fungi, where mycotransformation is well understood. Specific gene alterations can be designed and controlled via metabolic engineering. Metabolic control is shared by enzymes (i.e., enzymes are democratic). Mathematical modeling of metabolic control analysis can be used to make predictions as to how metabolic pathways will respond to manipulation. Fungal genes can be cloned to meet the objectives of mycoremediation. Fungal mutants that oversecrete specific enzymes can be produced, and various processes using such mutants may be designed and scaled up in the treatment of wastes and wastewaters. Fungal protoplasts can be exploited to enhance processes related to mycoremediation. At present, efforts to increase flux through specific pathways have met with limited

success. Potentially, the future of metabolic engineering is bright, but there is still a long way to go to understand this area of the metabolic network before the introduction of bioengineered yeast or fungi in the field of mycoremediation.

The first complete eukaryotic genome belongs to the yeast *Saccharomyces cerevisiae* (Dujon, 1996). The yeast genome contains 12 067 266 base pairs with 16 chromosomes and 6275 genes. The genome sequence has laid a strong foundation for work in the disciplines of agriculture, industry, medicine, and remediation. In a white paper for fungal comparative genomics, the Fungal Genome Initiative (FGI) Steering Committee identified a coherent set of 44 fungi as immediate targets for sequencing (Birren et al., 2003). Several projects have released information on the genome sequences of the yeasts *Schizosaccharomyces pombe* and *Candida albicans* and the filamentous fungi *Aspergillus nidulans*, *Aspergillus fumigatus*, *Neurospora crassa*, and *Coprinus cinereus*. The 13.8 million base pair genome of *S. pombe* consists of 4940 protein coding genes, including mitochondrial genome and genes (Wood et al., 2002). Ten thousand genes are predicted in the 40-Mb genome in the sequence of the first filamentous fungus, *N. crassa* (Galagan et al., 2003). The 30 million base pair genome of the first basidiomycete, *Phanerochaete chrysosporium* strain RP78, has been sequenced using a whole-genome shotgun approach (Martinez, et al., 2004). The genome reveals genes encoding oxidases, peroxidases, and hydrolytic enzymes involved in wood decay. This opens up new horizons related to the process of biodegradation of lignin and organopullutants and in the area of mycoremediation. Recently, yeast has been engineered with a binding affinity to cellulose (Nam et al., 2002). Genes encoding the cellulose binding domain (CBD) from cellobiohydrolase I (CBHI) and cellobiohydrolase II (CBHII) of *Trichoderma reesei* have been expressed on the cell surface of *Saccharomyces cerevisiae*.

REFERENCES

Acharya, C., L.B. Sukla, and V.N. Mishra (2005) Biological elimination of sulphur from high sulphur coal by *Aspergillus*-like fungi. *Fuel* **84**: 1597–1600.

Amiri-Eliasi, B., and C. Fenselau (2001) Characterization of protein biomarkers desorbed by MALDI from whole fungal cells. *Anal. Chem.* **73**: 5228–5231.

Arai, H. (2000) Foxing caused by fungi: twenty-five years of study. *Int. Biodeterior. Biodegrad.* **46**: 181–188.

Aynsley, M., A.C. Ward, and A.R. Wright (1990) A mathematical model for the growth of mycelial fungi in submerged culture. *Biotechnol. Bioeng.* **35**: 820–830.

Barlocher, F. (1992) Research on aquatic hyphomycetes: historical background and overview. In: *The Ecology of Aquatic Hyphomycetes*, F. Barlocher, ed. Springer-Verlag, Berlin, pp. 1–15.

Baronian, K.H.R. (2004) The use of yeast and moulds as sensing elements in biosensors. *Biosens. Bioelectron.* **19**: 953–962.

Bartnicki-Garcia, S. (1990) Role of vesicles in apical growth and a new mathematical model of hyphal morphogenesis. In: *Tip Growth in Plant and Fungal Cells*, I.B. Heath, ed. Academic Press, San Diego, CA, pp. 211–232.

Bartnicki-Garcia, S., F. Hergert, and G. Gierz (1989) Computer simulation of fungal morphogenesis and the mathematical basis for hyphal (tip) growth. *Protoplasma* **153**: 46–57.

Bennett, J.W., W.J. Connick, Jr., D. Daigle, and K. Wunch (2001) Formulation of fungi for in situ bioremediation. In: *Fungi in Bioremediation*, G.M. Gadd, ed. Cambridge University Press, Cambridge, pp. 97–112.

Bergauer, P., P.-A. Fonteyne, N. Nolard, F. Schinner, and R. Margesin (2005) Biodegradation of phenol and phenol-related compounds by psychrophilic and cold-tolerant alpine yeasts. *Chemosphere* **59**: 909–918.

Beuchat, L.R. (1992) Media for detecting and enumerating yeasts and moulds. *Int. J. Food Microbiol.* **17**: 145–158.

Birren, B., G. Fink, and E. Lander (2003) *Fungal Genome Initiative: A White Paper for Fungal Comparative Genomics.* Center for Genome Research, Cambridge, MA.

Borneman, J., and R.J. Hartin (2000) PCR primers that amplify fungal rRNA genes from environmental samples. *Appl. Environ. Microbiol.* **66**: 4356–4360.

Boswell, G.P., H. Jacobs, F.A. Davidson, G.M. Gadd, and K. Ritz (2003) Growth and function of fungal mycelia in heterogeneous environments. *Bull. Math. Biol.* **65**: 447–477.

Bradner, J.R., and K.M.H. Nevalainen (2003) Metabolic activity in filamentous fungi can be analyzed by flow cytometry. *J. Microbiol. Methods* **54**: 193–201.

Bredberg, K., B.E. Andersson, E. Landfors, et al. (2002) Microbial detoxification of waste rubber material by wood-rotting fungi. *Bioresour. Technol.* **83**: 221–224.

Breuil, C., L. Rossignol, and J.N. Saddler (1990) Refinement of an enzyme-linked immunosorbent assay for detecting sapstain fungi in wood. *Biotechnol. Tech.* **4**: 263–268.

Bridge, P., and B. Spooner (2001) Soil fungi: diversity and detection. *Plant Soil* **232**: 147–154.

Bumpus, J.A., M. Tien, D. Wright, and S.D. Aust (1985) Oxidation of persistent environmental pollutants by a white rot fungus. *Science* **224**: 1434–1436.

Cahill, D.M., and A.R. Hardham (1994) A dipstick immunoassay for the specific detection of *Phytophthora cinnamomi* in soils. *Phytophthology* **84**: 1284–1292.

Caldwell, I.Y., and A.P.J. Trinci (1973) The growth unit of the mould *Geotrichum candidum*. *Arch. Mikrobiol.* **88**: 1–10.

Ci, Y.-X., J. Feng, Z.-W. Jiang, and D.-Z. Luo (1997) The voltammetric behavior of *Saccharomyces cerevisiae*. *Bioelectrochem. Bioenerg.* **43**: 293–296.

Clausen, C.A. (1997) Immunological detection of wood decaying fungi: an overview of techniques developed from 1986 to the present. *Int. Biodeterior. Biodegrad.* **39**: 133–143.

Clausen, C.A., F. Green III, and T.L. Highley (1990) Early detection of brown-rot decay in southern yellow pine using immunodiagnostic procedures. *Document IRG/WP/2356*. International Research Group on Wood Preservation, Stockholm, Sweden.

Clausen, C.A., F. Green III, and T.L. Highley (1991) Early detection of brown-rot decay in southern yellow pine using immunodiagnostic procedures. *Wood Sci. Technol.* **26**: 1–8.

Cohen, R., and Y. Hadar (2001) The roles of fungi in agricultural waste conversion. In: *Fungi in Bioremediation*, G.M. Gadd, ed. Cambridge University Press, Cambridge, pp. 305–334.

Cox, P.W., G.C. Paul, and C.R. Thomas (1998) Image analysis of the morphology of filamentous micro-organisms. *Microbiology* **144**: 817–827.

Crittenden, P.D., and N. Porter (1991) Lichen-forming fungi: potential sources of novel metabolites. *Trends Biotechnol.* **9**: 409–414.

Crognale, S., F. Federici, and M. Petruccioli (2002) Enhanced separation of filamentous fungi by ultrasonic field: possible usages in repeated batch processes. *J. Biotechnol.* **97**: 191–197.

Cronenberg, C.C.H., S.P.P. Ottengraf, J.C. van den Heuvel, F. Pottel, D. Sziele, K. Schugerl, and K.H. Bellgardt (1994) Influence of age and structure of *Penicillium chrysogenum* pellets on the internal concentration profiles. *Bioprocess Eng.* **10**: 209–216.

Cui, Y.Q., W.J. Okkerse, R.G. van der Lans, and K.C. Luyben (1998) Modeling and measurements of fungal growth and morphology in submerged fermentations. *Biotechnol. Bioeng.* **60**: 216–229.

Daniel, G., and T. Nilsson (1990) Use of the enzyme-linked immunoassay (ELISA) and immunocytochemistry to detect soft-rot in preservative treated and untreated wood. *Document IRG/WP/2347*. International Research Group on Wood Preservation, Stockholm, Sweden.

Daniel, G., J. Jellison, B. Goodell, A. Paszczyncki, and R. Crawford (1991) Use of monoclonal antibodies to detect Mn(II)-peroxidase in birch wood degraded by *Phanerochaete chrysosporium*. *Appl. Microbiol. Biotechnol.* **35**: 674–680.

Diakumaku, E., A.A. Gorbushina, W.E. Krumbein, et al. (1995) Black fungi in marble and limestones: an aesthetical, chemical and physical problem for the conservation of monuments. *Sci. Total Environ.* **167**: 295–304.

Dighton, J. (2003) *Fungi in Ecosystem Processes*. Marcel Dekker, New York.

Dix, N.J., and J. Webster (1995) *Fungal Ecology*. Chapman & Hall, London.

Dorge, T., J.M. Carstensen, and J.C. Frisvad (2000) Direct identification of pure *Penicillium* species using image analysis. *J. Microbiol. Methods* **41**: 121–133.

Dubey, A.K., C. Suresh, S.U. Kumar, and N.G. Karanth (1998) An enzyme-linked immunosorbent assay for the estimation of fungal biomass during solid-state fermentation. *Appl. Microbiol. Biotechnol.* **50**: 299–302.

Dujon, B. (1996) The yeast genome project: What did we learn? *Trends Genet.* **12**: 263–270.

El-Shafei, H.A., N.H.A. El-Nasser, A.L. Kansoh, et al. (1998) Biodegradation of disposable polyethylene by fungi and *Streptomyces* species. *Polym. Degrad. Stab.* **62**: 361–365.

Filion, M., M. St.-Arnaud, and S.H. Jabaji-Hare (2003) Direct quantification of fungal DNA from soil substrate using real-time PCR. *J. Microbiol. Methods* **53**: 67–76.

Fujita, S.I., Y. Senda, S. Nakaguchi, et al. (2001) Multiplex PCR using internal transcribed spacer 1 and 2 regions for rapid detection and identification of yeast strains. *J. Clin. Microbiol.* **39**: 3617–3622.

Galagan, J.E., S.E. Calvo, K.A. Borkovich, et al. (2003) The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature* **422**: 859–868.

Georgiou, G., and M.L. Shuler (1986) A computer model for the growth and differentiation of a fungal colony on solid substrate. *Biotechnol. Bioeng.* **28**: 405–416.

Gerin, P.A., Y. Dufrene, M.-N. Bellon-Fontaine, M. Asther, and P.G. Rouxhet (1993) Surface properties of the conidiophores of *Phanerochaete chrysosporium* and their relevance to pellet formation. *J. Bacteriol.* **175**: 5135–5144.

Gibbs, P.A., R.J. Seviour, and G. Schmid (2000) Growth of filamentous fungi in submerged culture: problems and possible solutions. *Crit. Rev. Biotechnol.* **20**: 17–48.

Glancy, H., and J.W. Palfreyman (1993) Production of antibodies to *Serpula lacrymans* and their application in immunodetection systems. *Document IRG/WP/ 93-10004*. International Research Group on Wood Preservation, Stockholm, Sweden.

Glancy, H., A. Bruce, D. Button, J.W. Palfreyman, and B. King (1989) Application of immunological methods to the analysis and detection of *Lentinus lepideus* Fr. *Document IRG/WP/1422*. International Research Group on Wood Preservation, Stockholm, Sweden.

Goodell, B., K. Yamamoto, J. Jellison, M. Nakamura, T. Fujii, et al. (1998) Laccase immunolabeling and microanalytical analysis of wood degraded by *Lentinus edodes*. *Holzforschung* **52**: 345–350.

Griffin, D.W., C.A. Kellogg, K.K. Peak, et al. (2002) A rapid and efficient assay for extracting DNA from fungi. *Lett. Appl. Microbiol.* **34**: 210–214.

Gu, J.-D., T.E. Ford, N.S. Berke, et al. (1998) Biodeterioration of concrete by the fungus *Fusarium*. *Int. Biodeterior. Biodegrad.* **41**: 101–109.

Harish Prashanth, K.V., K. Lakshman, T.R. Shamala, and R.N. Tharanathan (2005) Biodegradation of chitosan-graft polymethylmethacrylate films. *Int. Biodeterior. Biodegrad.* **56**: 115–120.

Haselwandter, K., and M.R. Ebner (1994) Microorganisms surviving for 5300 years. *FEMS Microbiol. Lett.* **116**: 189–194.

Hasija, S.K. (1994) Biodegradation by aquatic fungi. Presented at the: Fifth International Mycological Congress, Aug. 14–21, Vancouver, BC, Canada.

Haugland, R.A., N. Brinkman, and S.J. Vesper (2002) Evaluation of rapid DNA extraction methods for the quantitative detection of fungi using real-time PCR analysis. *J. Microbiol. Methods* **50**: 319–323.

Hawksworth, D.L. (1991) Presidential address 1990. The fungal dimension of biodiversity: magnitude, significance and conservation. *Mycol. Res.* **95**: 641–655.

Hayden, R.T., X. Qian, G.W. Procop, G.D. Roberts, and R.V. Lloyd (2002) In situ hybridization for the identification of filamentous fungi in tissue section. *Diagn. Mol. Pathol. B* **11**: 119–126.

Hofrichter, M. (1998) Fungal attack on coal: modification of hard coal by fungi. *Fuel Energy Abstr.* **39**: 171.

Hofrichter, M., and R.M. Fakoussa (2001) Microbial degradation and modification of coal. In: *Biopolymers,* Vol. 1, *Lignin, Humic Substances and Coal*, M. Hofrichter and A. Steinbuchel, eds. Wiley-VCH, Weinheim, Germany, pp. 393–430.

Hofrichter, M., and W. Fritsche (1997) Depolymerization of low-rank coal by extra-cellular fungal enzyme systems. 2. The ligninolytic enzymes of the coal-humic acid-depolymerizing fungus *Nematoloma frowardii* b19. *Fuel Energy Abstr.* **38**: 296.

Holker, U., S. Ludwig, T. Scheel, and M. Hofer (1999a) Mechanism of coal solubilization by the deuteromycetes *Trichoderma atroviride* and *Fusarium oxysporum*. *Appl. Microbiol. Biotechnol.* **52**: 57–59.

Holker, U., M. Polsakiewicz, T. Scheel, S. Ludwig, et al. (1999b) Coal as inductor of esterases in the deuteromycete *Trichoderma atroviride*. In: *Proceedings of the Fifth International Symposium of Biological Processing of Fossil Fuels*, Madrid, Spain, pp. 26–29 (abstr.).

Hrncirip, P., J. Nahlik, and J. Vovsik (2002) The BIOGENES system for knowledge-based bioprocess control. *Expert Syst. Appl.* **23**: 145–153.

Iiyoshi, Y., Y. Tsutsumi, and T. Nishida (1997) Polyethylene biodegradation by white rot fungi. In: *Proceedings of the Ninth International Symposium on Wood and Pulping Chemistry*, Montreal, Canada, pp. 38-1 to 38-4.

Jasalavich, C., A. Ostrofsky, and J. Jellison (2000) Detection and identification of decay fungi in spruce wood by restriction fragment length polymorphism analysis of amplified genes encoding rRNA. *Appl. Environ. Microbiol.* **66**: 4725–4734.

Jellison, J., and B. Goodell (1986) Production of monoclonal antibodies to fungal metabolites. *Document IRG/WP/1306*. International Research Group on Wood Preservation, Stockholm, Sweden.

Jellison, J., and C. Jasalavich (2000) A review of selected methods for the detection of degradative fungi. *Int. Biodeterior. Biodegrad.* **46**: 241–244.

Jesenka, Z., E. Pieckova, and D. Bernat (1992) Heat-resistant fungi in the soil. *Int. J. Food Microbiol.* **16**: 209–214.

Jones, C.L., C.T. Lonergan, and D.E. Mainwaring (1993) A rapid method for the fractal analysis of fungal colony growth using image processing. *Binary* **5**: 171–180.

Klun, U., J. Friedrich, and A. Krzan (2003) Polyamide-6 fibre degradation by a lignolytic fungus. *Polym. Degrad. Stab.* **79**: 99–104.

Kruszewska, H., A. Misicka, and U. Chmielowiec (2004) Biodegradation of DNA and nucleotides to nucleosides and free bases. *Farmaco* **59**: 13–20.

Laborda, F., M. Fernandez, N. Luna, and I.F. Monistrol (1997) Study on the mechanisms by which microorganisms solubilize and/or liquefy Spanish coals. *Fuel Process. Technol.* **52**: 95–107.

Laborda, F., I.F. Monistrol, N. Luna, and M. Fernandez (1999) Processes of liquefaction/solubilization of Spanish coals by microorganisms. *Appl. Microbiol. Biotechnol.* **52**: 49–56.

Lamar, R.T., and R.B. White (2001) Mycoremediation: commercial status and recent developments. In: *Proceedings of the Sixth International Symposium on In Situ and On-Site Bioremediation*, San Diego, CA, V.S. Magar, M.F. von Fahnestock, and A. Leeson, eds., Vol. 6, pp. 263–278.

Langvad, F. (1999) A rapid and efficient method for growth measurement of filamentous fungi. *J. Microbiol. Methods* **37**: 97–100.

Lawton, J.H., and C.G. Jones (1995) Linking species and ecosystems: organisms as ecosystem engineers. In: *Linking Species and Ecosystems*, C.G. Jones and J.H. Lawton, eds. Chapman & Hall, New York, pp. 141–150.

Li, Z.J., V. Shukla, K. Wenger, et al. (2002) Estimation of hyphal tensile strength in production-scale *Aspergillus oryzae* fungal fermentations. *Biotechnol. Bioeng.* **77**: 601–613.

Lochman, J., O. Sery, and V. Mikes (2004) The rapid identification of European *Armillaria* species from soil samples by nested PCR. *FEMS Microbiol. Lett.* **237**: 105–110.

Lopes, P.T.C., and C. Gaylarde (1996) Use of immunofluorescence to detect the fungus *Hormoconis resinae* in aviation kerosene. *Int. Biodeterior. Biodegrad.* **37**: 37–40.

Lopez-Isunza, F., C.P. Larralde-Corona, and G. Viniegra-Gonzalez (1997) Mass transfer and growth kinetics in filamentous fungi. *Chem. Eng. Sci.* **52**: 2629–2639.

Lord, N.S., C.W. Kaplan, P. Shank, C.L. Kitts, and S.L. Elrod (2002) Assessment of fungal diversity using terminal restriction fragment (TRF) pattern analysis: comparison of 18S and ITS ribosomal regions. *FEMS Microbiol. Ecol.* **42**: 327–337.

Maheshwari, R., G. Bharadwaj, and M.K. Bhat (2000) Thermophilic fungi: their physiology and enzymes. *Microbiol. Mol. Biol. Rev.* **64**: 461–488.

Martinez, D., L.F. Larrondo, N. Putnam, et al. (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. *Nat. Biotechnol.* **22**: 695–700.

Matsuura, S. (2002) Colony patterning and collective hyphal growth of filamentous fungi. *Physica A* **315**: 125–136.

Matsuura, S., and S. Miyazima (1993) Colony of the fungus *Aspergillus oryzae* and self-affine fractal geometry of growth fronts. *Fractals* **1**: 11–19.

Maurice, C., and A. Lagerkvist (2000) Using *Betula pendula* and *Thelephora caryophyllea* for soil pollution assessment. *J. Soil Contam.* **9**: 31–50.

Mendes, B., P. Urbano, C. Alves, J. Morais, et al. (1998) Fungi as environmental microbiological indicators. *Water Sci. Technol.* **38**: 155–162.

Mille-Lindblom, C., E. von Wachenfeldt, and L.J. Tranvik (2004) Ergosterol as measure of living fungal biomass: persistence in environmental samples after fungal death. *J. Microbiol. Methods* **59**: 253–262.

Mitchell, D.A., D.D. Do, P.F. Greenfield, and H.W. Doelle (1991) A semimechanistic mathematical model for growth of *Rhizopus oligosporus* in a model solid-state system. *Biotechnol. Bioeng.* **38**: 353–362.

Monkemann, H., U. Holker, and M. Hofer (1997) Components of ligninolytic system of *Fusarium oxysporum* and *Trichoderma atroviride*. *Fuel Process. Technol.* **52**: 73–77.

Morgan, P., C.J. Cooper, N.S. Battersby, et al. (1991) Automated image analysis method to determine fungal biomass in soils and on solid matrices. *Soil Biol. Biochem.* **23**: 609–616.

Nam, J.-M., Y. Fujita, T. Arai, et al. (2002) Construction of engineered yeast with the ability of binding to cellulose. *J. Mol. Catal. B* **17**: 197–202.

Nazar, R.N., E.J. Robb, and T. Volossiouk (1996) Direct extraction of fungal DNA from soil. In: *Molecular Ecology Manual*, 1.3.6, A.D.L. Akkermans, J.D. van Elsas, and F.J. de Bruijn, eds. Kluwer, Dordrecht, The Netherlands, pp. 1–8.

Nelson, C.D., R.N. Spear, and J.H. Andrews (2000) Automated image analysis of live/dead staining of the fungus *Aureobasidium pullulans* on microscopic slides and leaf surfaces. *BioTechniques* **29**: 874–880.

Nielsen, J. (1992) Modelling the growth of filamentous fungi. *Adv. Biochem. Eng.* **46**: 187–223.

Oh, K.-B., T. Nishiyama, E. Sakai, et al. (1997) Flow sensing in mycelial fungi. *J. Biotechnol.* **58**: 197–204.

Papagianni, M., M. Mattey, and B. Kristiansen (1999) The influence of glucose concentration on citric acid production and morphology of *Aspergillus niger* in batch and culture. *Enzyme Microb. Technol.* **25**: 710–717.

Park, E.Y., P.N. Anh, and N. Okuda (2004) Bioconversion of waste office paper to L(+)-lactic acid by the filamentous fungus *Rhizopus oryzae*. *Bioresour. Technol.* **93**: 77–83.

Perfettini, J.V., E. Revertegat, and N. Langomazino (1991) Evaluation of cement degradation induced by the metabolic products of two fungal strains. *Experientia* **47**: 527–533.

Pitt, D.E., and A.T. Bull (1982) Use of autoradiography to quantify aspects of fungal growth and starvation in submerged liquid culture. *Trans. Br. Mycol. Soc.* **78**: 97–104.

Pletsch, M., B.S. de Araujo, and B.W. Charlwood (1999) Novel biotechnological approaches in environmental remediation research. *Biotechnol. Adv.* **17**: 679–687.

Prosser, J., and A.P.J. Trinci (1979) A model for hyphal growth and branching. *J. Gen. Microbiol.* **111**: 153–164.

Quinche, J.-P. (1990) *Lycoperdon perlatum*, a fungus accumulating heavy metals and selenium. *Mycol. Helv.* **3**: 477–486.

Ralph, J.P., and D.E.A. Catcheside (1997) Transformations of low rank coal by *Phanerochaete chrysosporium* and other wood-rot fungi. *Fuel Process. Technol.* **52**: 79–93.

Robinson, P.M., and J.M. Smith (1979) Development of cells and hyphae of *Geotrichum candidum* in chemostat and batch culture. *Trans. Br. Mycol. Soc.* **72**: 39–47.

Roche, N., A. Venague, C. Desgranges, et al. (1993) Use of chitin measurement to estimate fungal biomass in solid state fermentation. *Biotechnol. Adv.* **11**: 667–683.

Ruhm, W., L. Kammerer, L. Hiersche, and E. Wirth (1997) The ^{137}Cs/^{134}Cs ratio in fungi as an indicator of the major mycelium location in forest soil. *J. Environ. Radioact.* **35**: 129–148.

Scheel, T., U. Holker, S. Ludwig, S. Schinke-Kissing, and M. Hofer (1999a) Expression of laccase and manganese peroxide in basidiomycetes in the course of humic

acid degradation. In: *Proceedings of the Fifth International Symposium on Biological Processing of Fossil Fuels*, Madrid, Spain, pp. 26–29 (abstr.).

Scheel, T., U. Holker, S. Ludwig, and M. Hofer (1999b) Evidence for and expression of a laccase gene in three basidiomycetes degrading humic acids. *Appl. Microbiol. Biotechnol.* **52**: 66–69.

Schmidt, O., and U. Moreth (1998) Detection of the dry rot fungus *Serpula lacrymans* by amplified ribosomal DNA restriction analysis. *Document 98-10245*. International Research Group on Wood Preservation, Stockholm, Sweden.

Shrivastava, J.N., V.K. Ghawana, and A. Kumar (1996) Biodegradation of wool by *Trichophyton simii* and *Aspergillus niger. Mycoses* **39**: 483–487.

Singh, H. (1978) Nutritional studies on two pathogenic fungi. M.Phil. thesis, Punjabi University, Patiala, India.

Singh, H. (1991) Role of yeasts and fungi in wastes and wastewaters treatment. Ph.D. dissertation, Greenwich University, Hilo, HI.

Smit, E., P. Leeflang, B. Glandorf, J.D. van Elsas, and K. Wernars (1999) Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Appl. Environ. Microbiol.* **65**: 2614–2621.

Smith, M.L., J.N. Bruhn, and J.B. Anderson (1992) The fungus *Armillaria bulbosa* is among the largest and oldest living organisms. *Nature* **356**: 428–431.

Soddell, J.A., and R.J. Seviour (1994) A comparison of methods for determining the fractal dimensions of colonies of filamentous bacteria. *Binary* **6**: 21–31.

Subrahmanyam, S., K. Shanmugam, V.M. Madhav, et al. (2000) Assessment of growth of *Fusarium solani* by cyclic voltammetry and possible bioanalytical applications. *Analyst* **125**: 2166–2168.

Terziyska, A., L. Waltschewa, and P. Venkov (2000) A new sensitive test based on yeast cells for studying environmental pollution. *Environ. Pollut.* **109**: 43–52.

van de Peer, Y., and R. De Wachter (1995) Fungal phylogeny. In: *Molecular Microbial Ecology Manual*, 3.3.4, A.D.L. Akkermans, J.D. van Elsas, and F.J. de Bruijn, eds. Kluwer, Dordrecht, The Netherlands, pp. 1–12.

van Elsas, J.D., G.F. Duarte, A. Keijzer-Wolters, and E. Smit (2000) Analysis of the dynamics of fungal communities in soil via fungal-specific PCR of soil DNA followed by denaturing gradient gel electrophoresis. *J. Microbiol. Methods* **43**: 133–151.

Viagrow, A., H. Glancy, J.W. Palfreyman, and B. King (1991) The antigenic nature of *Serpula lacrymans. Document IRG/WP/1492*. International Research Group on Wood Preservation, Stockholm, Sweden.

Viniegra-Gonzalez, G., G. Saucedo-Castaneda, G. Lopez-Isunza, et al. (1993) Symmetric branching model for the kinetics of mycelial growth. *Biotechnol. Bioeng.* **42**: 1–10.

Webb, J.S., M. Nixon, I.M. Eastwood, M. Greenhalgh, et al. (2000) Fungal colonization and biodeterioration of plasticized polyvinyl chloride. *Appl. Environ. Microbiol.* **66**: 3194–3200.

White, T.J., T. Brun, S. Lee, and J. Taylor (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications*. Academic Press, New York, pp. 315–322.

Willmann, G., and R.M. Fakoussa (1997) Extracellular oxidative enzymes of coal-attacking fungi. *Fuel Process. Technol.* **52**: 27–41.

Wood, V., R. Gwilliam, M.-A. Rajandream, et al. (2002) The genome sequence of *Schizosaccharomyces pombe. Nature* **415**: 871–880.

Wosten, H.A.B., M.-A. van Wetter, L.G. Lugones, et al. (1999) How a fungus escapes the water to grow into the air. *Curr. Biol.* **9**: 85–88.

Yamada-Onodera, K., H. Mukumoto, Y. Katsuyaya, et al. (2001) Degradation of polyethylene by a fungus, *Penicillium simplicissimum* YK. *Polym. Degrad. Stab.* **72**: 323–327.

Yang, H., R. King, U. Reichl, and E.D. Gilles (1992) Mathematical model for apical growth, septation, and branching of mycelial microorganisms. *Biotechnol. Bioeng.* **39**: 49–58.

Yonni, F., M.T. Moreira, H. Fasoli, L. Grandi, and D. Cabral (2004) Sample and easy method for the determination of fungal growth and decolourative capacity in solid media. *Int. Biodeterior. Biodegrad.* **54**: 283–287.

Zaremski, A., M. Ducousso, Y. Prin, and D. Fouquet (1998) Characterization of tropical wood-decaying fungi by RFLP analysis of PCR amplified rDNA. *Document 98-10251.* International Research Group on Wood Preservation, Stockholm, Sweden.

Ziegenhagen, D., and M. Hofrichter (1998) Degradation of humic acids by manganese peroxidase from the white-rot fungus *Clitocybula dusenii. J. Basic Microbiol.* **38**: 289–299.

Zyska, B. (1997) Fungi isolated from library materials: a review of the literature. *Int. Biodeterior. Biodegrad.* **40**: 43–51.

2

FUNGAL TREATMENT OF INDUSTRIAL WASTEWATERS

2.1   INTRODUCTION

There is growing concern around the world regarding environmental pollu-
tion caused by industrial wastewaters. The problem has caused tightened
global discharge regulations. Regulatory and government agencies have
focused on the remediation of wastewaters with reference to various treat-
ment systems. Commercial applications in the monitoring and treatment of
industrial waste streams continue to satisfy the licensing and permitting cri-
teria of agencies all over the world. In this millennium, the environmental
protection sector is concerned with the internalization of environmental
policy and integration of environmental protection measures. In 2000, direc-
tives of the European Commission were established to exercise influence on
the environmental regulations of member states and consequently on techni-
cal requirements. However, the U.S. Environmental Protection Agency (EPA)
had developed and implemented industrial wastewater regulations more than
a decade earlier.

In developed and developing countries, increased environmental aware-
ness made it possible to understand and recover valuable resources for reuse.
Recycling of resources has become an integral and viable economic activity
for humankind. This has resulted in an increasing interest in water reuse
because of the problems of water supply and increasing cost of disposal of
wastewaters. Further treatment of effluent is required before reuse. The
resulting water quality and toxicity must be monitored, requiring new

developments in biosensor technology. Despite these initiatives, public acceptance is low unless the water is passed through a natural step: for example, being returned to a river. Several books have been published on wastewater reuse and reclamation. Mann and Liu (1999) described wastewater minimization through water reuse, regeneration, and recycle, and through process changes and the design of distributed effluent-treatment systems. Wastewater minimization through mathematical optimization has also been discussed. Total site wastewater reuse and regeneration has been discussed by Zbontar and Glavic (2000).

2.2   ALTERNATIVE INDUSTRIAL WASTEWATER BIOREACTORS

Bioreactors for the treatment of wastewaters have been known since 1970; however, activated sludge processes were employed as early as the 1920s. The capacity for wastewater treatment is proportional to the total biomass of a reactor. Advances in microbiology, biochemistry, biotechnology, and reactor engineering have developed various types of wastewater bioreactors. A possible general biological growth systematics has been developed by Jianlong et al. (2000). Biochemical operations, stoichiometry and kinetics, modeling, and applications of suspended and attached growth reactors are outlined in *Biological Wastewater Treatment* by Grady et al. (1999). In addition, design guidelines for bioreactors (Rossi, 2001) and bioreactor development (Mulder et al., 2001) have been presented.

Stirred tanks are the most common bioreactors for submerged cultivation. Other types of reactors, such as packed beds, bubble columns, airlift tower loops, and fluidized beds, are also employed under certain conditions. Clogging is usually a problem in packed-bed reactors because of the use of agricultural wastes as inexpensive substrates and nutrients with a large quantity of solids. The same is also true for fermentation wastewaters. Sedimentation of solids occurs in bubble columns. In fluidized-bed and airlift tower loop reactors, solids can be suspended in the upflowing liquid. Several researchers have written reviews on biofluidization (Furusaki, 1988; Shuler and Kargi, 1992; Godia and Sola, 1995). Three-phase fluidized and airlift suspension reactors are used for aerobic and anaerobic wastewater treatment and for the cultivation of immobilized and pellet-forming microorganisms, animals, and plant cells (Schugerl, 1997). Several types of cell immobilization can be used: (1) attachment of the microorganism to the surface of the carrier, (2) colonization of a porous carrier by organisms, (3) immobilization in hydrogel, and (4) microbial pellets or granulated sludges.

An innovative process involving a gas-lift mobile bed and a circulating floating-bed reactor (CFBR) for wastewater treatment has been proposed (Lazarova and Manem, 1996). The three-phase CFBR bioreactor does not require any complex devices (resulting in easier effluent and airflow distribu-

tion, no primary settling, and no back washing). This bioreactor guarantees excellent synergy between hydrodynamic and biological performance. Barratt et al. (1997) employed the Chemox process for advanced oxidation of a variety of wastewaters. The rotating biological contactor (RBC) is commonly used as a wastewater treatment process. Commercially available cation complexing surfactants have been used in the remediation of wastewaters (Schwuger et al., 2001).

Membrane bioreactors (MBRs) have an important place in wastewater treatment (Peters et al., 2000). An MBR employs ultrafiltration or microfiltration membranes for complete retention of a biomass. The MBR Bio-Filt, based on an FM module for ultrafiltration and a bioreactor of modular design manufactured in multitank form, has been developed for the improvement of wastewater purification, with a variety of easily adaptable applications. Membrane applications in various aerobic and anaerobic schemes for wastewater treatment have been described by Visvanathan et al. (2000).

Environmental biotechnology related to monitoring, mobilizing, and mineralizing pollution has been described in a report by Walters and Robinson (1997). This technology includes a membrane bioreactor coupled to an activated sludge reactor to provide complete retention of sludge. Biofilter reactors have certain advantages and disadvantages. A new type of biofilter reactor, the biogenerator, addresses some of the disadvantages of biofilter reactors. These days, the performance of wastewater treatment plants has become a focus for performance evaluation, and it is important to optimize these plants by kinetics and modeling. Flocculation, granulation and biofilms, and video imaging are also used to measure biofilm thickness in situ. Biosensors have potential applications in the monitoring processes of wastewater treatment, measuring the status of habitats in situ, and studying microbial gene regulation. A biological oxygen demand (BOD) biosensor is applied that uses immobilized yeasts and their modifications for effluents from wastewater treatment plants (Tanaka et al., 1994). Combined wastewater treatment may achieve the goal of remediation.

2.3   FUNGAL TREATMENT OF INDUSTRIAL WASTEWATERS

Fungal treatment of wastewaters dates back to the 1960s. Yeasts and fungi are used extensively to reduce the strength of a wide variety of food-processing wastewaters with concomitant production of food or fodder yeasts and fungi. The most commonly used yeast is *Candida utilis*, because of its ability to utilize a wide variety of carbon and nitrogen sources, its capacity to grow rapidly in high yields, and its tolerance of low pH. Fungi Imperfecti have the ability to convert biodegradable organic matter into a mycelium that not only has a high enough protein content to be valuable as an animal feed supplement, but also forms flocs that can be recovered readily by simple filtration

and screening. Some aspects of fungal treatment of industrial wastewaters have been highlighted by Nandan and Raisuddin (1992).

Several case histories of wastewater treatment by fungi have been described during the past decades. Lemonade-processing wastewater can be assimilated by yeasts (Hang, 1980). The amount of *Saccharomyces fragilis* growth parallels the BOD reduction of the wastewater. The settleability of *S. fragilis* is found to depend on temperature and age. Three strains of *Rhodotorula* have the ability to utilize (+)-limonene as a sole carbon source in the form of vapor (Thanh et al., 2004). These yeasts appear to have a coenzyme Q_{10} system. *C. utilis* has been used in the treatment of sauerkraut waste (Hang et al., 1973). The yeast is capable of removing approximately 80%, 70%, and 90% of the waste BOD, nitrogen, and phosphorus, respectively, in 16 hours. The addition of 0.05% sodium hydrogen phosphate resulted in increased utilization of brine components in the aerobic fermentation of pickle process brine by *C. utilis* (Stevenson et al., 1979). Supplementation with sodium phosphate reduced the BOD to 91%.

Celite is used as a carrier for fungi, but clogging occurs as a result of excessive growth and fluffy biofilm formation. This is controlled by phosphate limitation in fungi. Low spore concentration, a dilute medium, and low shear stress favor pellet formation. Pellet-forming fungi can be cultivated in aerated fluidized beds, bubble columns, or suspended airlift loop reactors. However, pellets can grow excessively at a low energy dissipation rate. Above a critical pellet size, depletion of oxygen leads to cell death, resulting in the formation of hollow pellets. Fungal mycelium is attached to the particle or penetrates the particle in the presence of solid substrates and forms pelletlike conglomerates. The substrate is utilized during growth and the pellets turn into filamentous mycelia. Hairy pellets are produced, forming a viscous suspension similar to filamentous fungi at a low energy dissipation rate. A multistage anaerobic reactor of 650 to 700 m^3 working volume has also been used for the treatment of baker's yeast wastewater (Markl and Reinold, 1994; Reinold et al., 1996).

Despite the discussion of different types of wastewater treatment by fungi reported elsewhere in the literature, in this chapter only the latest knowledge on specific wastewaters of important industrial types and their treatment by fungi is presented.

2.3.1   Starch-Processing Wastewater

2.3.1.1   Background.   Starch is a polysaccharide that occurs widely in plants in the form of reserve food material. Starch-processing wastewater is produced in large quantities, especially from food-processing plants all over the world. When discharged to lakes and rivers, the high organic load in the processing wastewater is a big concern relative to water quality. Most earlier studies reported the sterilization of materials employed as substrates. These processes require aseptic conditions and a retention time of more than 1 day. All these factors contribute to increased cost. About 25 to 30×10^3 tons of

sago, a principal cheap source of starch, is exported from Malaysia annually (Shim, 1992). Solid and liquid sago-processing wastes have been depicted diagrammatically by Vikineswary et al. (1994).

2.3.1.2 Composition and Characteristics. The production of sago-processing wastewater can reach as much as $1000 \, m^3$ per day in an average industry. A large amount of starch and pith residue is responsible for the chemical oxygen demand (COD) and biological oxygen demand (BOD) in sago wastewater (Chew and Shim, 1990). The cassava-processing industry generates wastewater that is known as *manipueira*. The wastewater has a pH of 5.5, 60000 mg/l COD, 38 g/l reducing and 20.2 g/l nonreducing sugars, and several minerals (Damasceno et al., 2003). Vermicelli factories discharge wastewaters containing high concentrations of starch and protein with high COD and BOD, respectively. The composition of synthetic starch wastewater has been noted by Fujita et al. (1993). This acidic starch-processing wastewater contains high carbohydrate (2360 to 3550 mg/l), starch (1520 to 2580 mg/l), and sugar (0.65 to 1.18%) contents along with high COD (11970 to 18900) and BOD (7980 to 12800 mg/l) and phosphate, sulfate, total Kjeldahl-N, and other constituents (Jin et al., 1999a). Proper treatment of such wastewaters is imperative.

2.3.1.3 Bioreactors and Fermentation. Yeast strains can reduce the wastewater strength of potato-processing wastewater along with the recovery of biomass (Karim and Sistrunk, 1985). Fermentation with the yeast *Saccharomyces fibuliger* ATCC 9947 effectively reduces COD in lye- and steam-peeled wastewaters. Vermicelli wastewater has also been treated using an acid-tolerant starch-degrading yeast (Hu, 1989). A semicontinuous culture removed about 92% COD at an appropriate hydraulic retention time (HRT) and solid retention time. A mixed culture of *Candida utilis* and *Endomycopsis fibuliger* efficiently assimilated both starch and free sugars in cassava starch effluent (Manilal et al., 1991). This treatment resulted in the reduction of about 94% of COD and 91% of BOD. An effective model has been proposed for continuous and two-stage fermentation with a pure amylolytic yeast first stage and a mixed culture second stage with a faster-growing nonamylolytic yeast (Pasari et al., 1989). *Geotrichum fragrans* utilizes sugars, thus reducing 40% of the COD of cassava wastewater (Damasceno et al., 2003). A process for high-lat treatment of predominantly carbohydrate-containing wastewater is discussed in a U.S. patent (Chigusa and Matsumaru, 1991).

The formation of pellets by *Aspergillus niger* ATCC 6275 and its applications to starch wastewater treatment have been examined (Fujita et al., 1993). One experiment was employed for pellet formation and a second was evaluated for a continuous starch wastewater treatment. Two reactors attached in series had the same configuration, with aeration and separation volumes of 1.1 L and 0.9 L, respectively. The pellets exhibited a 90% ability for starch decomposition and 75% for TOC removal, thereby proving effective in starch

wastewater treatment. The sago hampas can be subject to fungal degradation (Shim, 1992). Sago fibers and starch remaining in the hampas are degraded by a thermophilic fungus, *Myceliophthora thermophila*. During solid-state fermentation of sago hampas using *M. thermophila*, the highest cellulolytic and amylolytic activities on raw starch were 1300 IU/kg dried sago hampas and 1800 IU/kg dried sago hampas, respectively, after 72 hours. The final protein content of the upgraded product was not high; however, an improvement of more than 200% was achieved.

A strain of *Aspergillus oryzae* produces a fungal biomass protein and α-amylase in starch-processing wastewater (Jin et al., 1998). A one-stage non-aseptic airlift bioreactor removed 95% of the COD, 93% of the BOD, and 98% of the suspended solids and can be employed for agricultural irrigation. Starch-processing wastewater was treated with *Rhizopus oligosporus* in a laboratory-scale bench system using a 45-L airlift bioreactor with a working volume of 3.5 L (Jin et al., 1999a). Aeration in the range 1.0 to 1.7 (v/v) per minute was regulated to maintain the dissolved oxygen (DO) above 50% of saturation. The process proved efficient for the removal of complete suspended solids and a 96% reduction in COD. The economic aspects do not include pretreatment, but require nutrient supplementation together with nonaseptic operating conditions (Jin et al., 1998, 1999a). Starch hydrolysis occurs rapidly, at a rate of 0.19 g/l per hour for 10 hours and slows down afterward. An internal airlift loop reactor with a ceramic honeycomb support for biofilm (IAL-CHS) with *Candida tropicalis* is used in high-carbohydrate wastewater treatment (Zhang et al., 2005). This immobilized reactor accumulates dense and large amounts of biomass and produces a significant amount of single-cell protein (SCP). The carbohydrates are fermented to organic acid, thus lowering the pH and causing COD reduction.

2.3.1.4 *Enzyme Treatment.* Starch-processing wastes can be amenable to conversion into food, feed, or nonfood products with added value. The use of enzymes and enzymatic processing to reduce food wastes and to clean up food waste streams has been summarized by Shoemaker (1986). Amylases are polysaccharide hydrolases that are employed in the simultaneous saccharification and fermentation of starch and treatment of starch-processing wastewaters. Amylases can also be used for the production of alcohol from rice-processing wastewaters. α-Amylase and glucoamylase are also used in the production of photodegradable and biodegradable plastics (Coleman, 1990). The process entails the conversion of starch contained in potato waste to biodegradable plastics. Potential applications of enzymes in food-processing wastes have been discussed by Karam and Nicell (1997).

Glucoamylase is one of the most important enzymes used in food processing and in the commercial production of glucose from starch. This enzyme has the ability to hydrolyze starch almost completely into glucose. The fungal glucoamylases have been reviewed by Manjunath et al. (1983) and Norouzian et al. (2006). Many fungi produce glucoamylases under different fermentation

conditions, but species of *Aspergillus* and *Rhizopus* are widely employed in their commercial production. Recently, the amylolytic enzymes (i.e., α-amylase and glucoamylase from the thermophilic fungus *Thermomyces lanuginosus* strain ATCC 34626) have been purified (Nguyen et al., 2002). However, no information is available regarding the production of glucoamylase from wastes. Enzymes have a limited future in wastewater treatment. However, the full potential of enzymes has not been realized and a number of issues (e.g., identification and characterization of reaction by-products, disposal of reaction residues, and reduction of costs) still need to be addressed. Additional research will also be required to determine the suitability of enzymes under particular conditions and to optimize the enzymatic process as a whole.

2.3.1.5 *Production of Fungal Protein.*

In one report, about 1.55 to 2.0 g/l of the dry weight of centrifuged yeast solids from fermented steam-peeled potato wastewater was recovered after 6 to 28 hours of fermentation, which amounts to a crude protein content of 29 to 31% (Karim and Sistrunk, 1985). The recovered protein content of biomass was 22% (w/w) after 28 hours, which remained unchanged during 60 hours of fermentation (Manilal et al., 1991). Several possibilities for the utilization of sago-processing wastes have been recognized (Vikineswary et al., 1994). These include mushroom production, upgrading of animal feed, and production of algal biomass. The sago hampas can be used as raw material for the production of cellulose, xylanase, and raw starch–degrading enzymes during the preliminary submerged fermentation (Vikineswary and Nadaraj, 1992).

Fungal treatment of starch-processing wastewater can produce valuable products, such as the fungal biomass concomitant with purification of the effluent. The exponential growth of biomass was found to occur between 6 and 12 hours at a specific growth rate of 0.15 g/h (Jin et al., 1999a). The process resulted in the production of 4.5 to 5.2 g of dry biomass from 1 L of starch wastewater in 14 hours of cultivation. The fungal biomass contained a high (46%) protein content and was safe for human and animal consumption. This protein is marketable and the operating costs can be recovered. The glucoamylase activity in the culture filtrate increased proportionately with starch hydrolysis and biomass synthesis. Enzyme production remained unaffected by nutrient additions. This appears to be the first report regarding the production of glucoamylase from waste.

Growth pH, inoculum type and size, and superficial air velocity were found to have an effect on the mycelial morphology and fungal protein from starch-processing wastewater in submerged cultures by three strains of *Aspergillus oryzae* using an airlift bioreactor under nonaseptic conditions (Jin et al., 1999b). An inoculum technique was developed based on the morphology related to improved yield of fungal protein. The influence of inoculum quality on productivity, specific growth rate, and protein content reveals that the formation of small compact pellets favors the efficiency of biomass protein

production, harvesting, and better process operation. The yield of biomass protein was 6.1 g/l, which contains 38% protein, and 55 EU/ml of α-amylase from a 12-hour batch culture (Jin et al., 1998). The pellets favored easy harvesting of biomass protein. *Geotrichum fragrans* produced 12.8 g/l of biomass on cassava liquid waste after 12 hours (Damasceno et al., 2003). This cultivation resulted in the formation of several volatile compounds after 72 hours.

2.3.2   Dairy Industry Wastewater

2.3.2.1   Background.   The dairy industry is one of the largest single sources of food-processing wastewaters in the world. Significant impacts are caused by the cheese industry, due to release of its by-product, whey. Based on 13.5 million tons of cheese production in the world in 1993, release of whey can be calculated to be 121 million tons. The reports indicate that there is 50% global utilization of whey and that the rest is not disposed of properly. There are many well-documented articles in the literature on alternative methods of whey utilization, including yeast fermentation. The principal components in whey are lactose (44 to 52 g/l), proteins (6 to 8 g/l), and mineral salts (4.3 to 9.5 g/l) (Jelen, 1992). About 50% of the whey produced in the world every year is treated in wastewater treatment plants, discharged onto the land, or disposed of in lakes, rivers, or other water bodies. Whey is a significant source of organic pollution, with a high COD value of 50000 to 80000 mg/l and a BOD value of 40000 to 60000 mg/l, depending on the type of cheese. Lactose is responsible for 90% of whey's COD and BOD. Due to its high BOD level, the biological processes in wastewater treatment plants can be disrupted. In addition, continuous discharge of whey onto land can affect the properties of the soil, reduce crop yields, contribute to serious groundwater contamination, and eventually, pose a threat to human health and the environment.

Lactose cannot be assimilated by several microorganisms. Lactic acid bacteria can utilize it easily and lower the final pH, thus making it inhibitory for microbial growth. Lactose hydrolysis can be one of the solutions. At least 98 yeast species belonging to 19 genera can utilize lactose (Barnett et al., 1979). The utilization of lactose as a sole source of carbon and energy by yeasts usually requires a combination of lactose transport and lactase. However, strains of *Saccharomyces cerevisiae* contain lactase but are unable to utilize it, due to the lack of lactose transport (Barnett, 1981). Lactose metabolism and properties, and the regulation of lactose transport and lactases by yeasts, have been well summarized (Castillo, 1990). *Kluyveromyces fragilis* strains are employed for the production of yeast biomass from whey and can result in significant reduction of pollution load. In addition, large-scale processes have been in operation for the last several years for the production of *K. fragilis* biomass. Several researchers have reported a change in metabolism during aerated cultures of some *K. fragilis* strains. This change ranges from oxidation to mixed oxidation–fermentation, resulting in the formation of metabolic products such as alcohols, aldehydes, and esters.

2.3.2.2   Bioreactors and Modeling. Physical, chemical, and microbiological characterizations of dairy effluents collected periodically and their treatment in batches using free and alginate-entrapped *Candida pseudotropicalis* cells have been presented (Marwaha et al., 1989). Immobilized yeast cell treatment proves slightly better than that for free yeast cells. COD and BOD are found to be diluted to the Indian Standard Institution's safe levels by both systems. The biomass yield and COD removal efficiency of the fermentation process were studied in batches in shaken-flask and laboratory- and pilot-scale fermentors in the 1980s. Moresi et al. (1989) examined the scale-up of batch whey fermentation by *K. fragilis* to a 1000-dm^3 stirred fermentor by varying the stirrer speed, airflow rate, and initial concentration of lactose. A series of empirical regressions based on various operating conditions and geometric dissimilarity allow approximate but acceptable prediction of the stoichiometric and kinetic coefficients, thus confirming the capability of this model to scale up this fermentation process to production scale. The kinetics of continuous whey fermentation by *K. fragilis* has been studied in a 30-dm^3 stirred fermentor at different dilution rates and feed concentrations of lactose (Moresi et al., 1990). The concept of material and energy balances makes it possible to check the consistency of the steady-state responses of a chemostat culture on whey as well as to develop an unstructured model on a Monod-type kinetic equation for cell growth and two independent linear equations for lactose and oxygen consumption rates.

Oxygen transfer for lactic yeast production on whey substrates using an industrial airlift bioreactor has been modeled (Pigache et al., 1992). The fermentor consists of a 120-m^3 concentric-tube airlift reactor with a diameter of 3.80 m. Three empirical equations are used to express the Bunsen absorption coefficient *a*, Klag, and (Kla)r, and five parameters are used for the adjustment. The simulation displays an improvement in the fermentation process and calculations of the optimal operating conditions according to the production objectives. This tool can be applied as an operator guide and provides new perspectives for industrial fermentor design. The differences are exhibited in the growth kinetic behavior of *Torulopsis cremoris* in batch and continuous cultures (Cristiani-Urbina et al., 1997). The growth of *T. cremoris* on whey is described by a Monod equation. In a batch culture, *T. cremoris* shows a maximum specific growth rate 26% higher than that estimated in a continuous culture. This rate is higher than that of *K. fragilis*, which is employed in large-scale processes for biomass production from whey.

Yeasts have the ability to hydrolyze lactose in dairy effluents (Berruga et al., 1997). The disappearance of lactose in permeate is poorly correlated with intracellular lactase levels. *Candida kefir* NCYC 143 and *Kluyveromyces marxianus* NCYC 1548 cells show a maximum lactase activity of 200 nM ONP per minute 10 per cells. *K. marxianus* MTCC 1288 metabolized most of the lactose within 22 hours, to produce 8.8 g/l biomass and 2.10 g/l ethanol (Zafar and Owais, 2005). Bales and Castillo (1979) obtained an activity of 4 to 5 units on supplemented deproteinized whey. These fluctuations can also be

attributed to the reciprocal effects between lactase activity and both galactose (1.9 mM) and lactose (0 to 143 mM) concentrations in the permeate (Berruga et al., 1997).

Some bioreactors in whey/dairy effluents are noted in Table 2.1. Mixed yeast cultures are used to treat whey in batch and fed-batch fermentation under nonsterile conditions (Cristiana-Urbina et al., 2000). An airlift bioreactor with a total volume of 3.5 L is shown in Figure 2.1. The bioreactor column consists of Pyrex glass with a lower internal diameter of 7.7 cm, an upper internal diameter of 9.7 cm, and a total height of 72.4 cm. Of mixed yeast cultures, *Torulopsis cremoris* and *Candida utilis* produce the highest yield of biomass, with a value of 0.454 g of biomass per gram of lactose. *T. cremoris* produces extracellular metabolic products, such as organic acids, which are consumed by *C. utilis*. Moresi et al. (1989) found the whey lactose selectivity by *K. fragilis*, and when the carbon source was exhausted, the yeast utilized the intermediate compounds (ethanol, esters, glycerol, etc.) that had accumulated in the medium, thus exhibiting a diauxic growth pattern. A lactose–$(NH_4)_2SO_4$ ratio of 12 is optimal for cell yield (0.421 g/g), lactose consumption (100%), and COD removal (84%) without the addition of KH_2PO_4. Batch culture exhibits overall biomass yields of 0.349 and 0.459 g of biomass per gram of lactose for pure *T. cremoris* and mixed yeast cultures, respectively. The COD removal efficiencies are 87.13 and 92.68% for pure *T. cremoris* and mixed yeast cultures, respectively. The biomass yield obtained for a fed-batch culture of *T. cremoris* and *C. utilis* is greater than those for *K. fragilis* (Litchfield, 1983; Moresi et al., 1989), *K. marxianus* (Bainotti et al., 1987), and *T. cremoris* (Cristiani-Urbana et al., 1997); for mixed cultures of *K. fragilis–K. lactis–Torulopsis bovina* (Moulin et al., 1983; Pigache et al., 1992) and *Candida kefyr–C. valida* (Carlotti et al., 1991); and is similar to that of *K. fragilis* (Ghaly et al., 1992). The COD removal rate by this mixed culture is greater than those for *K. fragilis* (Moresi et al., 1990; Ben-Hassan and Ghaly, 1994) and *K. marxianus* (Bainotti et al., 1987). A new β-galactosidase–based biocatalyst has been developed by immobilization of whole *K. lactis* cells in calcium–alginate beads (Becerra et al., 2001). The

TABLE 2.1   Bioreactors in Whey and Dairy Effluents

Type of Bioreactor	Yeast or Fungus	Capacity of Bioreactor (L)	COD Reduction (%)	Lactose Hydrolysis (%)	Duration (hours)	Reference
Airlift (repeated fed-batch)	Mixed yeast cultures	3.5	96		60	Cristiana-Urbina et al., 2000
Immobilization (Ca–alginate beads)	*Kluyveromyces lactis* cells			99.5	30	Becerra et al., 2001
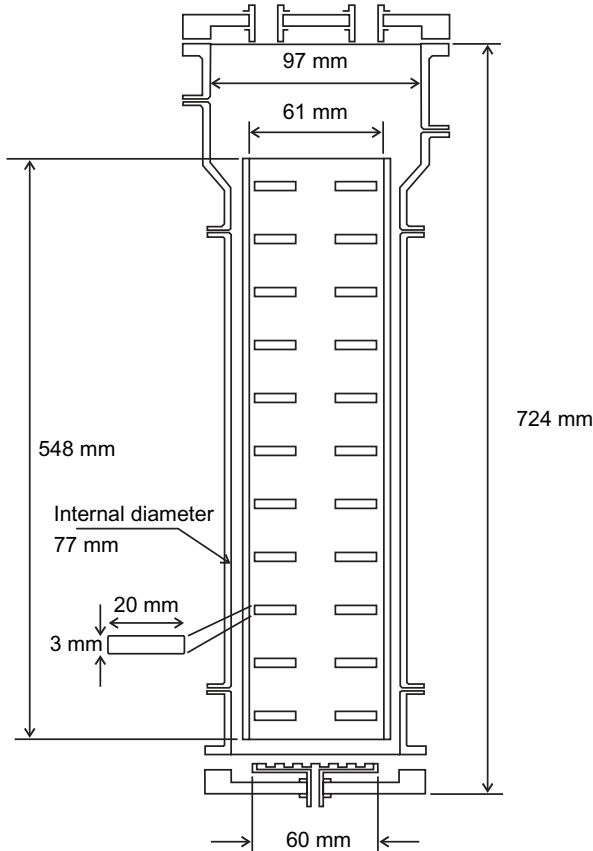
Figure 2.1 Airlift bioreactor for whey treatment by mixed yeasts. [Reprinted from Cristiana-Urbina et al. (2000), copyright © with permission from Elsevier.]

enzymatic activity was higher in alginate-immobilized biomass than in free-growing cells in the same medium. The permeabilized immobilized cells increased the hydrolysis rate of lactose: about 99.5% of milk-whey lactose at 30°C in 30 hours without ethanol production.

2.3.2.3 Production of Fungal Biomass. Production of yeast biomass, ethanol, and lactase or oils can be effective for the treatment of whey permeate, which reduces BOD by 90 to 95%. The production of yeast by fermenting the lactose in whey has been a subject of great interest, especially for Castillo (1990) and Kilara and Patel (1992). The majority of studies are focused on the lactic yeasts, mostly *K. fragilis*, which provides good biomass yield and acceptability as a safe microorganism. *K. fragilis* produces a higher biomass yield at a low concentration of lactose, implying an increase in the production

of metabolic products with increased lactose concentration (Moresi et al., 1989, 1990). Similar behavior is found in aerated cultures of *K. lactis* (Gonzalez Siso et al., 1996), *C. pseudotropicalis* (Bales and Castillo, 1979), *C. kefyr* (Carlotti et al., 1991), and *T. cremoris* (Cristiani-Urbina et al., 1997).

Several yeast species are known for the production of ethanol by lactose fermentation with more than 90% conversion efficiency. Several researchers have explored ethanol production by continuous fermentation with cell cycle or cell immobilization. Maiorella and Castillo (1984) presented process flow diagrams for the production of yeast biomass, lactase production, and ethanol production from whey. A schematic presentation of the entire whey process, various products and their applications, lactose manufacturing, and potential problems has been made by Marwaha and Kennedy (1988). Biogas, the biochemistry of methane production and energy analysis, ethanol production and various processes, and the production of organic acids, amino acids, gibberellic acid, polysaccharides, lipids, and flavor compounds from whey have also been discussed by Kilara and Patel (1992).

2.3.2.4 Lactases. Lactases can be employed in the hydrolysis of lactose in milk products, including whey, to reduce or eliminate problems associated with lactose intolerance in children and adults. Lactic yeasts are excellent sources of lactase, and these include *K. fragilis* (Sonawat et al., 1981), *K. lactis* (Gonzalez and Berry, 1982), *C. pseudotropicalis* (Itoh et al., 1982; Gomez and Castillo, 1983), *K. marxianus* (Goncalves and Castillo, 1981), and *Saccharomyces anamensis* (Banerjee et al., 1982). The optimum conditions for lactase production do not always coincide with those of biomass production. Fenton (1982) discussed solvent treatment for improved recovery of lactase. Many commercial lactase preparations are available in both soluble and immobilized form, but these are expensive for wastewater treatment. Lactases are also employed in dairy waste processing to produce value-added products (Shoemaker, 1986; Blasheck, 1992). Conversion of lactose from lactose hydrolysis in whey to L-ascorbic acid can be catalyzed by L-galactonolactone oxidase from the yeast *Candida norvegensis* (Shoemaker, 1986).

2.3.2.5 Genetics of Lactose Utilization. It is important to obtain stable strains of *Saccharomyces cerevisiae* to utilize lactose by genetic modification. The natural ability of *S. cerevisiae* to utilize lactose as a carbon source is solved by genetic manipulation following various strategies. The construction of a Lac$^+$ strain of *Saccharomyces* that carries the *LAC4* and *LAC12* genes of *K. lactis*, encoding for a β-galactosidase and a lactose permease, respectively, was first reported by Sreekrishna and Dickson (1985). This strain grows poorly in a medium with lactose and is mitotically unstable. Another strain of *Saccharomyces* grows in a medium with lactose by coexpression of the *Escherichia coli lacZ* gene and the *S. cerevisiae GAL4* gene (Perro et al., 1992). Cell lysis can occur due to overexpression of *GAL4*. Lac$^+$ strains of *S. cerevisiae* are also constructed based on expression of secretable β-galactosidase encoded

by the *lacA* gene of *Aspergillus niger* (Kumar et al., 1992). These strains grow in a medium with lactose, but the Lac phenotype is unstable and the utilization of sugar subjects it to diauxic growth (Ramakrishnan and Hartley, 1993).

A diploid strain of *S. cerevisiae* MRY286 is constructed that can assimilate lactose with high efficiency (Rubio-Texeira et al., 1998). This genetically stable strain carries multiple copies of *K. lactis LAC4* and *LAC12* genes that encode for a β-galactosidase and a lactose permease, respectively. A similar approach is employed to construct a Lac$^+$ industrial strain of baker's yeast that can be propagated in whey (Adam et al., 1999). To find Lac$^+$ strains of *S. cerevisiae* with optimal performance, strains differing in dosage of *LAC* genes have been constructed (Rubio-Texeira et al., 2000). A new strain is also being constructed that combines the Lac$^+$ phenotype with other traits of biotechnological interest, such as flocculence and the ability to assimilate starch.

2.3.3 Pharmaceutical Industry Wastewater

2.3.3.1 Background. Little attention is paid to the release of pharmaceutical substances exposed to the environment, which can also be recognized as environmental micropollutants. These are substances with known specific biological effects. Antibiotics employed as growth promoters or feed additives for a variety of animals often end up in the environment. Few studies are known that report results of medical substances in field samples other than those of sediment or treated wastewater. Several substances of pharmaceutical waste seem to persist in the environment. Current knowledge of the occurrence, fate, and effects of pharmaceutical wastes in the environment, including different exposure routes to the environment and legislation, has been described (Halling-Sorensen et al., 1998).

The activities of a pharmaceutical plant at Snackviken, Sweden, result in the discharge of approximately 1000 m^3/day of wastewater with a COD of 3 to 4 g/l. At another pharmaceutical plant, located at Gartuna, Sweden, the flow of wastewater is approximately 100 m^3/day with a COD of 0.8 to 1.5 g/l. At present, activated carbon is used to treat the most toxic portion (Rosen et al., 1998). The Swedish Environmental Protection Agency investigated the fact that wastewaters from the plants were highly toxic and contained large amounts of persistent organic material and phosphorus. Wastewater from the Snackviken plant was previously treated by evaporation in combination with reverse osmosis; today, it is incinerated. The final forms of the pharmaceutical wastes were characterized by Alvarino et al. (1997). Processes in formulation and preparation plants include the mixing of solid or liquid substances, pelletizing, encapsulating, and packing. These operational processes do not produce excessive wastes. The overall BOD of the entire waste is about 44 to 752 mg/l. The treatment includes pH control, 12-hour holding in an equalization basin, primary clarification, trickling filtration, and secondary clarification. Sludges are removed, dewatered, and disposed of in a landfill. The BOD of the final effluent is reduced to 30 mg/l.

Production of the majority of drugs leads to large variations in wastewater composition. Continuous development of new drugs and production methods also cause changes in the characteristics of wastewaters over time. Under such conditions it is necessary to examine and develop stable and cost-effective treatment methods for such wastewaters.

2.3.3.2   Process Development. In three pilot-scale tests, the multistage biofilm process showed the elimination of 90% of the COD and 95% of the total organic carbon (TOC) with better removal of toxicity (Rosen et al., 1998). The disadvantage of this process is that the pH must be adjusted to 4 in the fungal stages and then raised to 7. The methanogenic activity tests are also used for the determination of anaerobic biodegradability of some concentrated pharmaceutical wastes (i.e., waste biomass from threonine production, mycelium after *Penicillium* extraction, and excess activated sludge from the treatment of other pharmaceutical wastewaters) (Zabranska et al., 1994). Anaerobic biodegradability of each waste and mixture of wastes was evaluated. An optimum substrate concentration and methane yield for each waste and mixture were determined.

2.3.3.3   Conclusions. Organic material, phosphorus, and toxicity can be removed to a higher degree by the incorporation of fungal treatment. Treatability studies for the degradation of specific compounds in batch and continuous laboratory-scale processes are recommended before application in pilot-scale tests. When researching and developing new drugs and synthetic methods for compounds of pharmaceutical interest, it is important to consider the biodegradability of discharges to wastewaters.

2.3.4   Protein-Containing Wastewater

2.3.4.1   Background.   Large amounts of protein- and fat-containing wastewaters released from slaughterhouses and tanneries are discharged to the environment. Fungi can easily be employed in the treatment of such wastewaters. Biodegradation of fat into a yeast biomass can meet two beneficial objectives: (1) removal of fat from the wastewater and easier handling of the remaining waste for subsequent processing (e.g., extraction of soluble proteins or methane generation), and (2) use of the yeast biomass as a feed supplement. Since the 1970s, researchers have focused on the production of yeast cells using fat- or protein-containing wastewater. Oil from Atlantic menhaden is used to cultivate several types of yeasts (Burkholder et al., 1968). Fish oil (Hottinger et al., 1974) and stickwater, a by-product of the fish meal and oil industries (Green et al., 1976), are employed as substrates for *Candida lipolytica* and *Geotrichum candidum*, respectively. *Saccharomycopsis lipolytica* can grow on tallow or lard as a sole source of carbon and nitrogen (Tan and Gill, 1985).

About 3000 tons per month of wastewater produced by a company in Sweden contained approximately 6 to 8 g/l of fat and 10 to 13 g/l of protein (Rydin et al., 1990). Similar contents, 0.05% protein and 0.03% fat, in the wastewater from slaughterhouses, are also known (Grant, 1976). The fat contained 90% free fatty acids and the ash content included some iron and calcium. The composition of fatty acids in the wastewater was similar to the composition of tallow. It contained 43% oleic acid, 23% palmitic acid, 14% stearic acid, 7% linoleic acid, 4% palmitoleic acid, 2% myristic acid, and 2% linoleneic acid. The amino acid content was similar to that of gelatine.

2.3.4.2 Bioreactors. *Candida tropicalis* S001 grows and utilizes protein-containing wastewater (Rydin et al., 1990). A schematic process for bioconversion of fat- and protein-containing wastewater is shown in Figure 2.2. The cultures are performed in a 1.0- or 3.0-L fermentor with working volumes of 0.8 and 2.5 L, respectively. The fermentor cultures (batch and continuous) on wastewater are inoculated using a simple standard procedure at 800 rpm and on the model substrate (triolein) at 600 rpm under different environmental conditions. A 500-ml fluidized-bed bioreactor is employed for the production of methane generated from the protein phase of the wastewater. The maximum specific growth rate of *C. tropicalis* S001 increases in the order triolein <

WASTE WATER
protein & fat

↓

LOWERING OF pH

↓

(HEAT TREATMENT)

↓

YEAST CULTURE
pH = 4.0, 10% CO_2 in gas phase

↓

SEDIMENTATION

SOLUBLE PROTEIN   YEAST BIOMASS

↓   ↓

METHANE   FEED
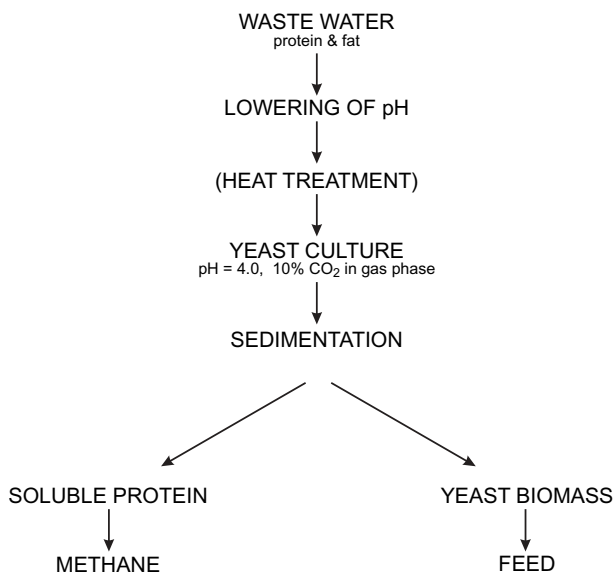
Figure 2.2 Bioconversion of fat- and protein-containing wastewater by *Candida tropicalis* S001. [Reprinted from Rydin et al. (1990), copyright © with permission from Springer Science and Business Media and the authors.]

glycerol < wastewater < glucose. The highest biomass yield (0.97 g dry weight/g fat) occurs on glycerol, followed by triolein (0.68 g dry weight/g fat) and glucose (0.57 g dry weight/g fat). In continuous cultures, the yeast growth rate coincides closely to that in batch cultures under similar conditions. The bioreactor reduces 86% of the COD using a feed of 40 kg COD/m^3 per day. The rate of methane production is 10.8 m^3/m^3 reactor per day, which constitutes a yield of 0.31 m^3 methane/kg COD_{red}. The growth of yeasts at a low pH generally provides a sterilizing effect in a continuous system for its domination. Most bacteria cannot grow or compete for growth at low pH. Several yeasts are well recognized to grow on lipids between pH 3.5 and 4.0 [e.g., *Candida lipolytica* (Hottinger et al., 1974), *S. lipolytica* (Tan and Gill, 1984), and *C. tropicalis* (Rydin et al., 1990)].

2.3.5 Oil Manufacturing Plant Wastewater

2.3.5.1 Background. Several physical and chemical processes using a dissolved flotation unit are established as a pretreatment for high concentrations of oil-containing wastewaters. The pretreatment usually requires a large quantity of chemicals, such as coagulants, acids, and alkalis, and the disposal of floating waste oil arising from the treatment process. The London Dumping Treaty, implemented in 1996, prohibits disposal of wastes in oceans. A biological process has been developed for the treatment of oils without pretreatment (Chigusa et al., 1996). This process also greatly reduced the generation of floating waste oil to be disposed of.

Wastewater from a soybean oil manufacturing plant was comprised of 88% oils, 3% carbohydrates, 3% proteins, and 6% unknown substances (Chigusa et al., 1996). The influent COD, BOD, and hexane extract concentrations range from 24 000 to 61 000 mg/l, 12 000 to 27 200 mg/l, and 6700 to 17 000 mg/l, respectively. The pH range of the wastewater is 6.5 to 7.5. Most of the oils are detected in the form of suspended solids, and these are between 5990 and 16 600 mg/l. Total nitrogen ranges from 401 to 497 mg/l.

In Malaysia, palm oil mill effluent (POME) is a major source of pollution, with an annual generation of 9 million tons (Chan et al., 1983). This effluent contains a high organic load and needs to be treated before discharge to any water body. The anaerobic fermentation of POME has been examined by some researchers (Sinnappa, 1979; Ibrahim et al., 1984). Anaerobic fermentation by bacteria takes more hydraulic retention time for the digestion of POME. However, some workers have discussed the roles of *Aspergillus oryzae* (Barker and Morgan, 1981) and *Penicillium chrysogenum* (Suwandi and Mohd, 1984) in the treatment of POME effluents. *Trichoderma viride* also plays a role in the treatment of POME (Karim and Kamil, 1989). The POME from a site in India contains about 250 000 mg/l COD, 11 000 mg/l BOD, 65 mg/l total dissolved solids, and 9000 mg/l of chloroform-soluble material (Oswal et al., 2002). Montet et al. (1983) used palm oil for the production of single-cell protein (SCP) by *Candida rugosa*.

2.3.5.2 Assay of Oil-Decomposing Ability. Two tests are used for evaluation of the ability of isolated fungi to hydrolyze and remove oil in effluents. The first test involved a study of lipase and β-oxidation activities with a synthetic medium containing 2% soybean oil as a sole source of carbon (Kou et al., 1983). Yeast strains are inoculated and incubated with shaking at 30°C. After 7 days, the hexane extracts of the broth are measured for weight, acid value, and saponification value. The residual free fatty acids and residual glycerides are calculated using equations (Japan Oil Chemists Society, 1971; Harada, 1986). A second test is employed to evaluate differences in hexane extract removal rates between soybean oil manufacturing plant wastewater and two synthetic media, one containing soybean oil and the other containing linoleic acid (the main component of soybean oil).

2.3.5.3 Bioreactors. Nine strains of yeasts are isolated from decomposing oil to treat soybean oil wastewater without pretreatment (Chigusa et al., 1996). An enrichment culture technique is employed for the isolation of yeasts. Of these yeast strains, seven belong to *Candida* genus, which is also selected as a high producer of lipase (Chen et al., 1990). N1 and N9 strains remove 65% and 78% of hexane extracts, respectively, as well as 94% of glycerides. This results in lower β-oxidation activity than lipase activity. On the other hand, seven other yeast strains exhibit higher β-oxidation activity. However, the mixture of all nine strains is superior to any single strain in both β-oxidation capability and lipase activity. Synergism is also noted in mixed strains of oil-decomposing yeasts. The removal rates are lowered in the order linoleic acid, soybean oil, raw wastewater. The optimal temperature range of yeast treatment in hexane extracts was 30 to 35°C and pH 2.5 to 6.5. A laboratory-scale reactor with a 50-L tank was inoculated with all oil-decomposing yeasts at 25°C without pH control. The reactor removed more than 98% of hexane extracts of the wastewater from a daily load of 0.5 kg of hexane extracts per kilogram of yeast and about 150 mg/l of hexane extracts remains. An activated sludge process was used as an aftertreatment to meet the sea target level of 30 mg/l at the time of design of the pilot plant (Chigusa et al., 1989).

Table 2.2 shows a flowsheet and operating conditions for a pilot plant used in the treatment of soybean oil wastewater for one year (Chigusa et al., 1996). The yeast reactor tank removes 99% of oils using isolated yeasts. The activated sludge process further treated the yeast-treated water and the hexane extract was reduced to 2 mg/l for discharge to the sea. Soybean oil decomposition by isolated yeasts without a dissolved flotation unit was demonstrated. The relationship between the quality of yeast-treated water and hexane extract loading shows the stability of the treated water at 100 mg/l within the range 0.30 to 0.75 kg of hexane extract per kilogram of yeast per day. Similar results have been achieved by yeast treatment in the dried food and fish meal industries (Tosaki et al., 1991; Nawamura et al., 1992). During pilot-plant operation, the yeast flora detect *Candida hellenica*, the most dominate species,

TABLE 2.2 Flowsheet and Operating Conditions of a Pilot Plant for the Treatment of Soybean Oil Wastewater

H_2SO_4
↓

influent → equalization tank → yeast reactor tank → yeast settling tank → activated sludge tank → final settling tank → effluent

⎣← return yeast ⎦   ⎣← return sludge ⎦

	Throughput (m³/d)	Capacity (m³)	Influent Hexane Extracts (mg/l)	Hexane Extract Load (kg/kg per day)	BOD Load (kg/kg per day)	Microorganism Concentration (mg/l)	Oxygen Demand (kg/kg BOD per day)	pH	Temperature (°C)
Yeast reactor tank	0.37–0.83	1.0	6670–17300	0.3–0.9	0.6–1.4	7000–16000	0.6	4.5–6.5	15–35
Activated sludge tank	0.37–0.83	1.5	54–198	0.01–0.03	0.1–0.3	1500–3000	1.0	5.8–8.6	15–35

Source: Chigusa et al. (1996), copyright © with permission from Elsevier.

followed by *C. schatavii* and *C. fluviatilis*. These species grow, forming mycelia/pseudomycelia, and become intertwined to form pellets. Such intertwined pellets of mycelia show a better sedimentation rate and can stabilize water quality in the yeast treatment. In addition, all strains in the reactor tank metabolize oil by a tricarboxylic acid cycle in which organic matter is oxidized and has a low fermenting ability.

Four types of treatments have been performed to study the fermentation of POME (Karim and Kamil, 1989): raw POME, boiled POME, raw POME with 5% inoculum of spores or mycelium separately, and boiled POME with 5% inoculum of spores or mycelium separately. More than 95% of the COD (COD 44 and 56 mg/l) was reduced with POME treated with spores or mycelium of *Trichoderma viride* at days 14 and 10 of the incubation period, respectively. This implies more effectiveness of the mycelial treatment in reducing the COD than of the sporal treatment. Biomass recovery with the mycelium and sporal inocula is 1.37 to 1.42 g/l (dry weight) after 10 days and 1.29 to 1.21 g/l (dry weight) after 14 days, respectively. Other researchers obtain better biomass yield on POME using filamentous fungi (Barker and Morgan, 1981; Suwandi and Mohd, 1984). The crude protein content from the fungal biomass of the treated POME ranges from 37.6 to 40.7% using sporal and mycelial inoculation after 10 and 14 days of incubation, respectively. Thus, fermentation of the POME by *T. viride* can be an alternative pretreatment in the reduction of the pollution strength of wastewater. In addition, fungus can compete against the indigenous microflora in raw POME, resulting in efficient reduction of COD. Treatment of POME by *Yarrowia lipolytica* NCIM 3589, a marine hydrocarbon-degrading yeast, reduces about 95% of the COD within 2 days (Oswal et al., 2002). A chemical coagulant further reduces the COD. A consortium developed from garden soil shows a clarification of effluent and a pH adjustment between 6 and 7. The complete treatment eliminates 99% of the COD, to 1500 mg/l.

2.3.6   Silage Wastewater

2.3.6.1   Background. During winter, controlled fermentation of crops of high moisture content, such as forage maize and grass, leads to the production of silage. Plant juices are expelled from the ensiled crop, and together with surface water, form silage wastewater. The National Rivers Authority (1992) reported silage effluent to be 300 times more polluting than raw domestic sewage. Snerik et al. (1977) stated four times faster degradation of silage effluent than sewage and can be 1000 times more potent as a pollutant.

Silage wastewater is considered as one of the most potent agricultural wastes, with a high BOD value. The pH of the silage effluent ranges from 3 to 5 and poses problems of corrosion to concrete and steel. Arnold et al. (2000) noted the compositions of three silage effluents. When discharged into water bodies, effluent 1, the most acidic effluent, can decrease pH and kill fish and other aquatic species. The impact of such pollution is even higher

when discharge occurs in small streams with low flow and low dilution rates. Scorching of grass or other crops results due to spreading of the effluent, followed by the depletion of oxygen in the surrounding soil. The effluent can find its way into the surface water or groundwater. Well-preserved silage effluent can be fed to animals, as it deteriorates in quality rapidly. Patterson and Kilpatrick (1991) reported feeding within 3 to 4 days after production or being stored anaerobically. Aerobic treatment of silage effluent (Deans and Svoboda, 1992; Galanos et al., 1995) results in a reduction in BOD and a change in pH. Farm waste pollution was described by Beck (1989). The application of membrane filtration to silage effluent has also been examined (Dunlea and Dodd, 1989). This is considered only a part of integrated waste facility treatment.

2.3.6.2 Legislation. In 1998, the Ministry of Agriculture, Fisheries and Food (1999) estimated the cutting of 42.3 million tons of forage crops for silage production in the UK, which resulted in an estimate of 2115 million liters of effluent. In dry years, effluent production is much lower. The variable production of effluent causes the difference in the number of incidents of total agricultural water pollution in wet years (25%) compared to dry years (14%) (Haigh, 1994). Information reveals 114 to 234 pollution incidents of silage effluent between 1995 and 1998 (UK Environment Agency, 1996, 1997, 1998, 1999), accounting for 7% of organic pollution incidents. In the UK, fines of up to £20000 or a 3-month prison sentence can be imposed for polluting water courses.

2.3.6.3 Growth of Fungi. Cultivation of the yeast *Torulopsis* (*Candida*) *glabrata* on grass silage effluent leads to an 85% reduction in carbohydrate content (Olsen and Pedersen, 1974). However, the yeast is dropped from silage treatment, due to its inability to utilize lactic acid and potential pathogenicity. Malt–yeast extract–sucrose agar (MYSA) is the medium of choice for the enumeration and isolation of fungi from silage (Skaar and Stenwig, 1996). *Penicillium roqueforti* is most prevalent on bales of grass silage in Ireland and represents 52% of all isolates (O'Brien et al., 2005). Of 11 isolates, *Candida utilis* and T2B (*Galactomyces geotrichum*) are effective in reducing the pollutants of silage effluents of variable age and composition (Arnold et al., 2000). A COD of 74 to 95% is reduced in all effluents, but the percentage reduction accounts for 95% in more the dilute and fresher effluents (effluents 2 and 3) (Table 2.3). Both fungi exhibit TOC removal of 60 to 87%, with the greatest reduction in effluent 2. Both yeasts assimilate both D- and L-lactic acids. Both yeasts remove 100% of L-lactic acid in 50% and 25% silage effluents, and both organisms remove 50 to 98% of D-lactic acid in 25% effluent and 90% for *C. utilis* and T2B, respectively, in 50% effluent. Both yeasts reduce varying degree of phenolic compounds in effluent 1. The increase in pH due to the removal of lactic acids and volatile fatty acids (VFAs) occurs in all treatments. Both yeasts are effective in 82 to 99% removal of phosphate

TABLE 2.3 Percentage COD, TOC, and Phosphate Reductions of Silage Effluents by T2B and *Candida utilis*

Effluent Type	COD (mg/l)				TOC (ppm)				µg PO$_4$P/ml			
	T2B		*C. utilis*		T2B		*C. utilis*		T2B		*C. utilis*	
	50%	25%	50%	25%	50%	25%	50%	25%	50%	25%	50%	25%
Effluent 1	79	N/A	74	N/A	78	N/A	60	N/A	97	N/A	>99	N/A
Effluent 2	83	86	66	93	81	87	74	86	>99	>99	>99	>99
Effluent 3	95	95	92	91	84	84	79	73	90	82	96	85

Source: Adapted from Arnold et al. (2000), copyright © with permission from Elsevier.
N/A, not applicable.

in both 25% and 50% dilutions of all silage effluents. *C. utilis* and T2B remove about 95 to 100% of acetic and propionic acids and 70 to 100% of the four higher VFAs.

2.3.6.4   *On-Farm Treatment.* The feasibility of a simple on-farm treatment process of silage effluent is presented by the addition of a large inoculum of yeast followed by vigorous aeration (Arnold et al., 2000). The dominating yeast T2B can survive and compete easily with the native microflora. The higher reductions in COD and TOC indicate the use of a yeast treatment process to protect human health and the environment. However, the process may not reduce the residual COD in the effluent sufficiently to discharge the effluent to a water course. Further treatment is required before final direct discharge. Biomass produced during this process can be considered for use as a fertilizer or soil conditioner.

2.3.6.5   *Production of Fungal Biomass.* The protein content for *C. utilis* is 160 mg/g dry weight and for T2B is 74 mg/g (Arnold et al., 2000). Elemental analyses of the biomass grown on effluent 2 result in higher concentrations of P and Ca in T2B than in *C. utilis* and very much higher concentrations of K in *C. utilis* than in T2B. In both yeasts, glutamate accounts for the highest amount: ca. 12 to 17 mol%. Alanine, leucine, and glycine each account for ca. 10% of the total amino acid content. Besides the low protein content, biomass reveals a good amino acid profile for use as an animal feed supplement.

2.3.7   Acidogenic Wastewater

2.3.7.1   *Background.* Highly loaded effluents containing 45% of the total organic industrial pollution are produced by the food industry. These effluents contain high concentrations of volatile fatty acids (VFAs), whose treatment requires many successive steps. To degrade the highly concentrated biodegradable contaminants, Laulan (1987) revealed that a yeast reactor, followed by an anaerobic bacteria reactor in series, can satisfy the effluent discharge requirements. A yeast reactor is known to be prone to contamination by bacteria, and an anaerobic bioreactor usually requires a long retention time. Elmaleh et al. (1996) proposed an alternative process of acidogenic reactor in series followed by a yeast reactor. This operational setup provides shorter retention times in the acidogenic reactor. The operation of the following yeast reactor can be performed at low pH to limit contamination by bacteria. Anaerobic acidogenesis of a complex wastewater has been discussed (Dinoupoulou et al., 1988). The main organic compounds include VFAs such as acetic acid, propionic acid, or butyric acid. Elmaleh et al. (1995) reported effluents produced by the organic chemical industry that contain a highly concentrated acetic acid solution. The synthetic effluent is composed of acetic acid, propionic acid, butyric acid, or a mixture of these acids and is employed as a sole source of carbon in the yeast reactor (Elmaleh et al., 1999). Maugeri

and Goma (1980) overviewed the single-cell protein (SCP) production from organic acids with *Candida utilis*.

2.3.7.2   Bioreactors and Modeling. A process using an anaerobic acido-genic reactor followed by a yeast (*C. utilis*) reactor in series to treat highly concentrated effluents is shown in Figure 2.3 (Elmaleh et al., 1999). The pilot units consist of two perfectly mixed reactors in series. The first comprises a 60-L mechanically stirred anaerobic acidogenic reactor, thermoregulated at 35°C and operated at a retention time of 35 hours. Its pH is maintained at 6
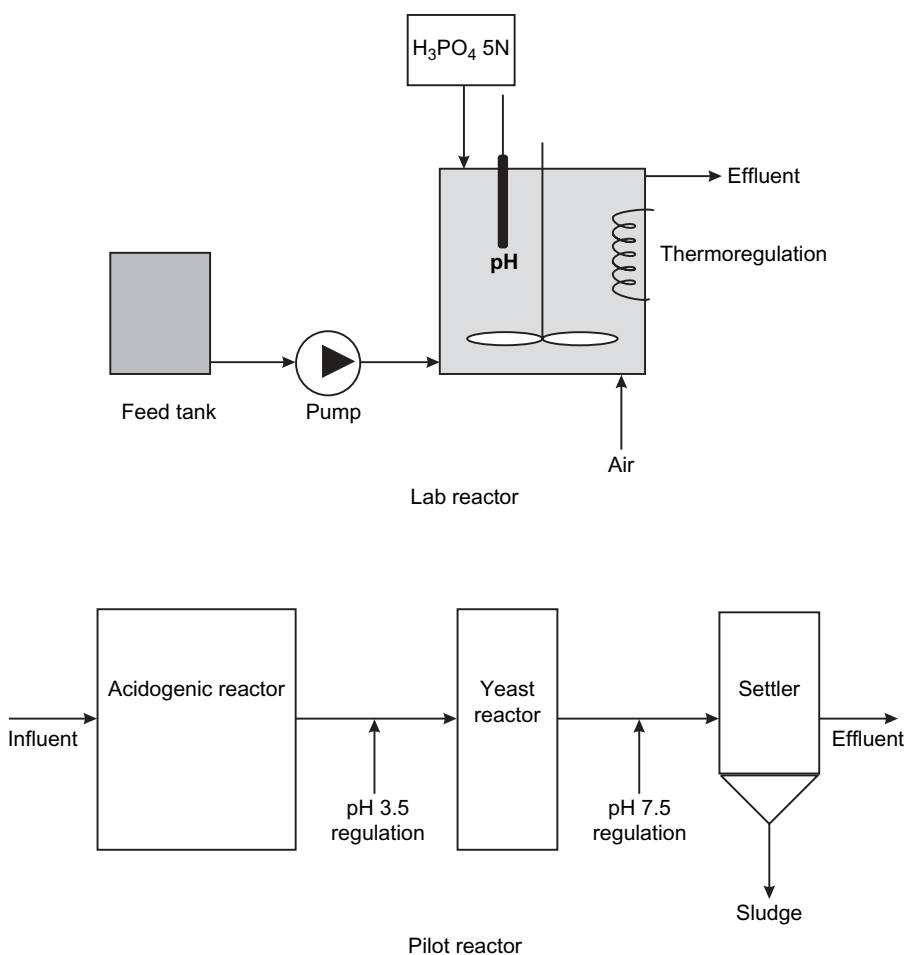
Figure 2.3   Experimental units (lab reactor and pilot reactor) for treatment of acidogenic wastewater using *Candida utilis*. [Reprinted from Elmaleh et al. (1999), copyright © with permission from Elsevier.]

to prevent product inhibition or lactic fermentation (Edeline, 1993). The following 16-L yeast reactor, also known as a pilot reactor, is receiving the effluent from the acidogenic reactor. Its pH is regulated at 3.5 and operated at 18 to 20°C. The pilot reactor is stirred mechanically at 600 rpm. Oxygen monitoring shows an oxygen concentration of higher than 80 to 90% of the saturation concentration. In a laboratory reactor, about 97% TOC (Table 2.4) is removed for all the organic acids and mixtures of different proportions (Elmaleh et al., 1999). The same is true for the pilot yeast reactor fed with acetic acid. Edeline (1993) reported 95% removal of VFAs if the acidogenic process is optimized in the pilot unit. The pilot reactor shows 87% TOC reduction when it is fed exclusively with the acidogenic reactor effluent. About 90% TOC is removed by two reactors in series and remains steady during a 6-month operation.

The growth kinetics of *C. utilis* on acetic acid has been studied in a laboratory reactor under both batch and continuous conditions (Elmaleh et al., 1996, 1999). In a batch reactor, the rate of growth gradually increases until acetate exhaustion. In a steady-state continuous reactor, the substrate is exhausted and the rate of growth depends on the concentration of residual acetic acid. A significant uncoupling between growth and substrate utilization is found in the batch reactor, and the transient state of the continuously stirred-tank reactor cannot be predicted. This implies the metabolic state of the yeast sensitive to substrate inhibition effects in a steady-state perfectly mixed reactor at low substrate concentration.

A Haldane-type inhibition model is proposed to describe the growth of *C. utilis* on sodium acetate as a sole source of carbon and energy (Cama and Edwards, 1970). Ko and Edwards (1975) employed a model to calculate the growth inhibition of *C. utilis* at high acetate concentrations. Jackson and Edwards (1975) used a model to incorporate the pH effects to model the specific growth rate of *C. utilis* on acetate. Assimilation of acetic acid by *C. utilis* is also proposed by an equation (Maugeri, 1980). Defrance (1993) investigated the performance and limitations of a yeast reactor for the treatment of wastes in the food industry and suggested an empirical equation to describe the growth inhibition of *C. utilis* on acetic acid. His model accounts for a

TABLE 2.4 Growth Kinetics of *Candida utilis* on Organic Acids

Fungus	Organic Acid	Type of Reactor or Fermentor	TOC Removal (%)	Reference
Candida utilis	Acetic, propionic, and butyric	Batch or continuous reactor	97	Elmaleh et al., 1996, 1999
C. utilis	Butyric	Biolafitte fermentor		Ortiz et al., 1997
C. utilis	Acetic	Culture reactor		Defrance, 1993

critical substrate concentration beyond which no biological activity can be observed. The critical substrate concentration is important for the design of a culture reactor in the presence of inhibitory substrates such as VFAs. However, little is known about models describing the degradation of other VFAs using yeasts. The kinetic behavior and mathematical modeling of *C. utilis* on butyric acid and ammoniac-nitrogen (NH_4^+—N) for the removal of carbon and nitrogen from wastewater have been described (Ortiz et al., 1997). The culture apparatus consists of cultivation in a 2-L Biolafitte fermentor containing 1.5 L of aerated Henry's medium mixed at 800 rpm. The data are fitted to the models using Bio-ASPRO, a software tool designed for bioprocess modeling and control. The maximum growth rate on butyric acid is optimal at 4.86 g/l, and the strongest inhibition occurs at 12.82 g/l. Acid inhibition is due to uncoupling of the pH gradient by passive diffusion across the cell membrane (Herrero, 1983) and acidification of the cytoplasm (Lawford and Rousseau, 1993). A concentration of 0.52 g/l of NH_4^+—N produces maximum yield (0.61 g/l) and productivity (0.57 g/l·h). Growth-limiting conditions occur at 0.81 g/l of undissociated butyric acid and 0.08 g/l of NH_4^+—N. Conversion of acid and NH_4^+—N into biomass is possible at their respective low concentrations in a reactor. Construction of a general expression to model the specific growth rate has been developed. More or less similar results in batch reactors depicting incomplete inhibition of the yeast growth using acetic acid as substrate with concentrations from 0.44 to 26.46 g/l at pH 6.0 and 30°C have been shown by Maugeri (1980). The undissociated acid concentration is 1.57 g/l from the total acetic acid concentration of 26.46 g/l. The undissociated acid concentration of the mixture (acetic, propionic, and butyric acids) is 2.4 g/l from a total mixture concentration of 40 g/l. The threshold inhibition values are two to three times higher than the threshold recorded by Ortiz et al. (1997). *C. utilis* shows more sensitivity to the undissociated acid concentration than other species of *Candida* and species of *Schizosaccharomyces*.

2.3.8 Olive Mill Wastewater

2.3.8.1 Background. Olives in Mediterranean countries, constitute about 98% of global production. Large quantities of olive mill wastewater (OMW) are produced during the manufacture of oil by traditional mill and press processes. This wastewater arises from the vegetation water of olives, washing and process water, and soft tissues from the olive pulp and oil, and forms a stable emulsion. Borja et al. (1992) reported a release of 2.5 L of waste per liter of oil produced. About 1 to 2 tons of OMW is produced during the production of 1 ton of olives (Garcia et al., 2000). In addition to its high polluting power, OMW usually exhibits a high level of phytotoxic and antibacterial activity, due to the presence of various phenolic compounds. Balice et al. (1988) reported that the antimicrobial qualities of OMW must be diluted prior to treatment, but that a strain of *Aspergillus niger* remains unaffected by the toxic substances in the OMW.

Conventional methods are not effective in the treatment of OMW. A good degree of success is achieved through the use of an electrolysis system (Israilides et al., 1997). Besides some advantages of previous work, the anaerobic digestion of OMW was accompanied by problems of high toxicity, low biodegradability of the effluent, and acidification of reactors. The presence of phenolic compounds slows the process, removal of part of the COD, and its economic viability. Aerobic biological methods appear to be suitable because these treatments tend to diminish the phenol content and reduce toxicity. Vitolo et al. (1999) discussed the treatment of olive oil wastes. Evaporation is used to separate the vegetation water into an aqueous liquid that can be purified by a biological process, and a residue where 98% of the organic load is concentrated.

Since the OMW contains the phenolics, tannin, and lignin, attention is focused on microorganisms capable of degrading such compounds. Many soil- and litter-inhabiting bacteria and fungi can partly degrade these compounds. Their metabolism leads to the formation of low-molecular-weight aromatic compounds that are found in OMW, and these can be further degraded. Species of *Aspergillus*, *Geotrichum*, and *Phanerochaete* possess high activities of catabolic enzymes and can utilize a wide variety of simple aromatic compounds. Microbial composition of OMW from four disposal ponds led to the identification of several species of bacteria, yeasts, and molds (Millan et al., 2000). Besides other species of fungi, strains of *Fusarium*, *Paecilomyces*, *Penicillium*, and *Scopulariopsis* exhibited a marked capacity for the detoxification of OMW, removing its antibacterial activity completely.

2.3.8.2   *Composition and Characteristics.* Garcia et al. (2000) obtained OMW from a continuous olive-processing operation that contained a COD of 82000 mg/l and a total phenol content of 1200 mg/l. The composition of OMW varies widely depending on the type of process (batch or continuous) involved in obtaining oil. The compositions of the OMW have also been featured (Martin et al., 1991; Vassilev et al., 1997; Fountoulakis et al., 2002). A phenolic content of 1.2 to 10 g/l is reported by some researchers (Moreno et al., 1987; Vinciguerra et al., 1995; D'Annibale et al., 1999). The debittering process of green olives (GOW) also produces wastewater rich in polyphenolics, high COD, and alkalinity (Aggelis et al., 2002).

2.3.8.3   *Fermentation.* Fungal fermentation of the OMW is noted in Table 2.5. *Phanerochaete flavido-alba* decolorizes OMW in static or semistatic cultures at 30°C (Blanquez et al., 2002). The experiments were conducted in a Bioflo III bioreactor. The toxicity of the culture medium was eliminated up to 70%, as determined by a Microtox system. The fungus produces manganese-dependent peroxidase (MnP) and laccase, which are able to decolorize and reduce the aromatic compounds and toxicity simultaneously. A strain of *Phanerochaete chrysosporium* isolated from Moroccan OMW has the ability to degrade OMW under different culture conditions (Kissi et al., 2001). *P.*

TABLE 2.5 Fungal Fermentation of Olive Mill Wastewater

Fungus	Dilution of the OMW	Phenolic Reduction (%)	COD Reduction (%)	TOC Reduction (%)	Decolorization (%)	Biomass Yield (g/l dry weight)	Duration (hours/days/months)	Reference
Phanerochaete flavido-alba	Undiluted	51			70		14d	Blanquez et al., 2002
Phanerochaete chrysosporium	50%	60	60		60		9d	Kissi et al., 2001
Penicillium strain P4	Undiluted	54	61		80	21.5	20d	Robles et al., 2000
Pleurotus ostreatus	Undiluted	52			49		1m	Aggelis et al., 2002
P. ostreatus	Sterilized 50%	78	10				21d	Fountoulakis et al., 2002
	Thermally processed 50%	67	12				19d	
P. ostreatus	Undiluted	65	9				21d	Martirani et al., 1996
	10%	90					100h	
Phanerochaete chrysosporium	Undiluted	92	75	75			150h	Garcia et al., 2000
Aspergillus niger		76	73	73			115h	
Aspergillus terreus		64	63	67			112h	
Lentinus (Lentinula) edodes	20%	90		85	72		12d	Vinciguerra et al. 1995
Pycnosporus coccineus	Diluted at 50g/l		47		58	12.32	20d	Jaouani et al., 2003
Pleurotus sajor-caju			50		72	8.05		
Coriolopsis polyzona			42		75	3.47		
Lentinus (Lentinula) tigrinus			39		50	6.72		

chrysosporium eliminates more than 50% of the color and phenols from OMW within 6 days, compared to similar results reached in 12 days by *Pleurotus ostreatus*. Of seven strains of *Penicillium* isolated from wastewater disposal ponds, strain P4 produces the best biomass: 21.5 g dry weight per liter on undiluted OMW after 20 days (Robles et al., 2000). This and other strains eliminate a high amount of COD and phenolic content of OMW and cause an increase in pH.

Of eight white-rot fungi, *P. ostreatus* shows a high decolorization efficiency of 49% (Aggelis et al., 2002). All fungi reveal the highest activity of laccase, followed by manganese-independent peroxidase (MIP). Substantial MnP activity is found only in GOW treated with *P. ostreatus* and *Abortiporus biennis*; LiP and veratryl alcohol oxidase (VAOx) are not detected. Early detection of laccase activity correlates with the final amount of reduction of total phenolics. MnP is necessary for efficient decolorization. *Pycnosporus coccineus*, *Pleurotus sajor-caju*, *Coriolopsis polyzona*, and *Lentinus* (*Lentinula*) *tigrinus* were good in decolorization and COD removal of crude olive mill wastewaters diluted to 50 and 75 g/l after 20 days (Jaouani et al., 2003). *P. sajor-caju* and *C. polyzona* showed 72 to 75% decolorization of effluent diluted to 50 g/l COD. Different mechanisms seemed to be involved, due to differences in enzyme activity. *Panus tigrinus* efficiently removed phenols, color, and organic load from OMW at an initial soluble COD value of 43 000 mg/l (D'Annibale et al., 2004b).

Aerobic pretreatment of OMW with *P. chrysosporium* reduces the phenolics and toxic aromatic compounds followed by anaerobic treatment (Gharsallah et al., 1999). Pretreatment of crude OMW by *P. chrysosporium* reduced the COD from 107 g/l to 55 g/l and to 85 g/l after sedimentation. About 70% of color and 80% of COD were removed when the cultures were supplemented with veratryl alcohol and flushed with oxygen (Sayadi and Ellouz, 1992). Glutamate suppresses the biodegradation system. Decolorization by *Aspergillus niger* occurs due to fungal adsorption, including 55.5% degradation of phenols and 73.5% degradation of tannins (Hamdi et al., 1991a). *A. niger* removed 61.6% COD in flasks and 52.5% in a fermentor after 72 hours. Hamdi et al. (1991b) optimized fermentation by *A. niger*. The highest biomass and greatest COD removal were obtained with a ratio of COD removed to $N:SO_4$ of 100 to 3:1.5. The fungus fermented the OMW without dilution. A novel method combining a strain of *A. niger* and electrochemical treatment in the presence of H_2O_2 has been developed for the treatment of green table olive–processing wastewater (Kyriacou et al., 2005). The fungal treatment removed the COD and selected phenols by 66 to 86% and 65%, respectively. Nearly 96% COD and measured phenols were eliminated during electrochemical treatment with 2.5% H_2O_2. The pilot plant removed 75% COD with 1.6% H_2O_2. Coagulation with $Ca(OH)_2$ led to 98% overall removal efficiency for the combined treatment.

The phenol detoxifying activity of *P. ostreatus* is concomitant with increasing phenol oxidase production (Martirani et al., 1996). The phenol oxidase

activity was 1.1 U/ml (first cycle), 2.0 U/ml (second cycle), and 2.6 U/ml (third cycle) after 70 hours of each cycle. Four species of *Pleurotus* produced a high yield of fruiting bodies under certain conditions (Sanjust et al., 1991). Species of *Pleurotus* produced large amounts of laccase in the medium, thus significantly reducing the concentration of phenolics and other toxic compounds. A highly significant correlation between color reduction, TOC, and total phenols using *Lentinus* (*Lentinula*) *edodes* has been noted (Vinciguerra et al., 1995). NMR analysis confirms bioconversion of phenolic and aliphatic compounds in the form of signals of metabolic products due to activity of the fungus. Exoenzyme production such as phenol oxidase was maximum after 7 days and MnP was detected on the seventh day. Such results confirm involvement of the lignin-degrading system of the fungus.

2.3.8.4 Modeling. Fermentation follows Monod kinetics either on COD or TOC as the substrate and produces values for maximum specific growth and growth yield (Garcia et al., 2000). A comparison among various fungi using kinetic information indicated the removal of phenolic compounds from OMW in the sequence *P. chrysosporium* > *A. niger* > *A. terreus*. A kinetic study was conducted of the anaerobic digestion of OMW previously fermented with *Geotrichum candidum*, *Azotobacter chroococcum*, and *Aspergillus terreus* (Borja et al., 1995). A batch-fed bioreactor containing sepiolite was employed as a supporting material for the mediating bacteria. The anaerobic process followed the first-order kinetics. The kinetic constant increased with a decrease in phenolic compound content and biotoxicity of the pretreated OMWs. The yield coefficient was 260 (untreated OMW), 300 (*Geotrichum*-pretreated OMW), 315 (*Azotobacter*-pretreated OMW), and 350 (*Aspergillus*-pretreated OMW) ml of CH_4 STP per gram of COD. Borja et al. (1993) also described kinetic study of OMW previously fermented aerobically with *A. terreus* using the same bioreactor and method. The process exhibited no inhibition phenomena, and the total phenolic compounds and biotoxicity was reduced by 94.3% and 86.8%, respectively. An average removal of COD fraction was 70%, and the yield coefficient was 349 ml of CH_4 STP per gram of COD, 30% higher than for untreated OMW.

2.3.8.5 Immobilization. Immobilized bioreactors for the decolorization of the OMW are listed in Table 2.6. OMW has been decolorized by agitated submerged cultures of free and immobilized *P. chrysosporium* (Sayadi et al., 1996). No decolorization of OMW was found when *P. chrysosporium* was grown in the form of pellets. Decolorization was obtained after removing the high-molecular-weight polyphenolic fraction (>60 kDa) by ultrafiltration. The highest decolorization (85%) and COD removal (50%) occurred in a medium producing high lignin peroxidase (LiP). Extensive depolymerization and subsequent accumulation of phenolics of intermediate molecular weight were found. The application of *P. chrysosporium* immobilized on polyurethane foam in repeated batches resulted in more than 70% color and COD removals

TABLE 2.6 Immobilized Bioreactors for Decolorization of Olive Mill Wastewater

Immobilization	Bioreactor System	Fungus	Decolorization (%)	Phenol Removal (%)	Duration (hours/ days)	Reference
Polyurethane foam	Repeated-batch fermentation	*Phanerochaete chrysosporium* cells	70		85h	Sayadi et al., 1996
Polyurethane sponge cubes	Repeated-batch fermentation	*Aspergillus niger* cells		67	8d	Vassilev et al., 1997
Ca–alginate beads	Batch fermentation	*Candida tropicalis* cells	60	36.5	24h	Ettayebi et al., 2003
Chitosan and glutarldehyde	Batch fermentation	*Lentinula edodes* laccase		67	24h	D'Annibale et al., 1999
	Fluidized-bed reactor		50	42	2h	
Oxirane	Fluidized-bed reactor	*Lentinula edodes* laccase	22	70	2h	D'Annibale et al., 2000

from the OMW. Three cycles can be achieved using immobilized bioreactor without sporulation or lysis. OMW was treated by immobilized cells of *Aspergillus niger* in polyurethane sponge cubes to produce a material of low toxicity and its enrichment with soluble rock phosphate (RP) to formulate a suitable process for fertilizer (Vassilev et al., 1997). Three types of medium were studied: OMW + N, OMW + N + RP, and OMW + RP. Five-cycle repeated batch fermentation process indicated the production of the highest fungal biomass of 5.32 g per gram of carrier on a medium containing both N and RP. Reduction of phenols was registered in all treatments. Vassilev et al. (1996) reported the influence of the buffering capacity of RP on the acid-producing activity of immobilized *Penicillium variable* P16. RP solubilized with a maximum level of soluble P of 0.58 g/l during the fourth batch cycle of the OMW + RP treatment. This application results in three types of waste liquid that can be used further for various purposes.

Laccase of *Lentinula edodes* immobilized on chitosan by adsorption and subsequent cross-linking with glutarldehyde eliminated total phenols and *ortho*-phenols by 67% and 72%, respectively, after 24 hours (D'Annibale et al., 1999). Removal of the *para*-substituents 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid occurred at a slower rate than the removal of *ortho*-diphenolic compounds. Treatment on six consecutive batches showed significant removal of color, total phenols, and *ortho*-phenols. Significant regain (60%) of the immobilized activity was restored by washing the column with three bed volumes of 0.1 M McIllvaine buffer, suggesting the possibility of further use of the immobilized catalyst. Oxirane-immobilized *Lentinula edodes* laccase treatment operated in a batch recirculation mode removed about 70% of total phenols, 88% of *ortho*-phenols, and 22% decolorization of the OMW in 2 hours (D'Annibale et al., 2000). Less than 5% of 3,4-dihydroxyphenylethanol, catechol, 3,4-dihydroxycinnamic acid, 3,4-dihydroxyphenylacetic acid, and 3,4-dihydroxyphenylpropionic acid was recovered from the OMW. The dephenolization efficiency in a fluidized-bed bioreactor was reduced in successive batches and was restored using acetate buffer.

2.3.8.6 Enzyme Treatment. A reduction in total phenolics exceeded 50% during treatment of GOW with purified laccase from *Polyporus pensitius* (Aggelis et al., 2002). This was not followed by a decline in phytotoxicity. This may be attributed to the formation of phenoxy radicals and quinonoids, which repolymerize in the absence of VAOx. OMW was treated with purified phenol oxidase from *Pleurotus ostreatus* (Martirani et al., 1996). Incubation of OMW with different amounts of phenol oxidase catalyzed in an undetectable transformation. This can be attributed to the fact that these wastewaters exist in the form of a suspension, rather than in a solution consisting of insoluble heterogeneous polymers such as carbohydrates, proteins, lipids, or phenol polymers (Sainz-Jimenez and Gomez-Alarcon, 1986). The raw OMW inhibited the activity of phenol oxidase by adsorption or binding to polymeric

substances. Treatment of ultrafiltered OMW with polyphenol oxidase reduced 90% of phenolic compounds compared to untreated OMW.

Lignin peroxidase (LiP) and manganese peroxidase (MnP) of *Phanerochaete chrysosporium* contribute to decolorization of OMW (Sayadi and Ellouz, 1995). About 25% of the OMW was decolorized when *P. chrysosporium* grows in the presence of a high Mn(II) concentration in which a high level of MnP ($0.65\,\mu$M) is produced. More than 70% of the OMW was decolorized when *P. chrysosporium* grows in the presence of a low Mn(II) concentration that results in a high level of LiP ($0.3\,\mu$M). The highest levels of OMW decolorization occurred at a low initial COD level in conjunction with high levels of extracellular LiP. The positive effect of veratryl alcohol on LiP activity indicates the high levels of OMW decolorization.

Potential applications of oxidative enzymes and phenol oxidase–like compounds in wastewater treatment have been described (Duran and Esposito, 2000). A reduction in treatment cost can be achieved through enzyme immobilization. Tyrosinase catalyzed the hydroxylation of phenols and dehydrogenation of *ortho*-diphenols, which can result in excellent removal of phenols in an immobilized form. Laccase eliminated phenols through a polymerization process. Peroxidases, including lignin peroxidase (LiP), manganese peroxidase (MnP), and horseradish peroxidase (HRP), were also discussed.

2.3.8.7   Toxicity Testing.   Some aspects of toxicity after fungal treatment have been discussed in the foregoing paragraphs. No difference in toxicity was found when tested on *Bacillus cereus* due to phenol oxidase treatment of OMW, despite a 90% reduction in phenolic compounds (Martirani et al., 1996). This could be due to the formation of reaction products of phenol oxidase: for example, oxidative coupling polymers and quinonoids, which are more inhibiting than the original substances. Capasso et al. (1995) reported the generation of *ortho*-benzoquinone by laccase-catalyzed oxidation of catechol, which completely inhibited the growth of *Pseudomonas syringae* pv. *savastanoi* and *Corynebacterium michiganense* at $500\,\mu$M concentration. However, antibacterial activity was expressed by OMW on these bacteria up to a dilution of 1:4.

The soil nitrogen-fixing bacterium *Rhizobium* sp. strain 1230 has also been employed to test toxicity before and after the treatment of OMW with immobilized laccase for 6 and 24 hours (D'Annibale et al., 1999). The experiments indicated growth suppression of this strain in a 1:4 dilution of OMW. The growth suppressive effect was also found at 1:8 diluted OMW without laccase treatment, and this effect was removed by laccase incubation for 24 hours before dilution. Borja et al. (1997) related the impact of selected phenolics from OMW toward the kinetics of acetoclastic methanogenesis to the molecular structure of phenols. Higher inhibitory effects of *ortho*-phenols were exhibited than for *para*-coumaric and *para*-hydroxybenzoic acids. A higher degree of toxicity was expressed by cinnamic acid derivatives than by benzoic acid derivatives. However, D'Annibale et al. (1999) demonstrated the highest

removal rates of *ortho*-phenols and the cinnamic acid derivative *para*-coumaric acid by immobilized laccase from *Lentinula edodes*, causing reduced toxicity of OMW. *L. edodes* removed phenols from OMW and significantly reduced the OMW phytotoxicity, as shown by durum wheat (*Triticum durum* Desf.) germinability (D'Annibale et al., 2004a). The toxicity of pretreated OMW could be attributed to the concentration of high-molecular-weight aromatics, which remained high after fungal treatment (Gharsallah et al., 1999). Thus, OMW can be used in agriculture after the removal of phytotoxic effects.

Phytotoxicity was not eliminated during treatment of GOW by *Pleurotus ostreatus* despite a 76% reduction of the phenolics (Aggelis et al., 2002). Fermentation of OMW with *Penicillium* P4 caused no antibacterial activity against *Bacillus megaterium* ATCC 25848 (Robles et al., 2000). The toxicity of the OMW was determined by a luminescent bacteria *Photobacterium phosphoreum* test using a Microtox system (Borja et al., 1993). The EC_{50} was 1.24 (toxic units 80.8) prior to inoculation with *Aspergillus* and 9.43 (toxic units 10.6) upon fermentation. *Phanerochaete flavido-alba* reduced the phenol and lipid contents of semisolid olive mill waste mixtures and pine-chip mixtures, resulting in a substrate with lower antibacterial and phytotoxic effects (Linares et al., 2003).

2.3.8.8 Economic Importance. During the past two decades, a number of fungi have been studied for converting OMW into new value-added products (Table 2.7). One of them is the production of single-cell protein (SCP) from olive black water. OMW as raw material for SCP using *A. niger* under nonoptimized conditions has been evaluated (Hamdi et al., 1991a). The soluble protein in the filtrate from flasks and a fermentor was 3.75 g/l and 4.95 g/l, respectively. A good prospect exists for the exploitation of OMWs for mushroom cultivation (Zervakis et al., 1996). Some important characteristics of mushroom cultivation, such as earliness, quality of basidiomata, and yield, were noted. The optimal concentration of OMW for mycelia growth of *Pleurotus* was assessed for the production of biomass in liquid media.

TABLE 2.7 Prospects of Economic Importance of Olive Mill Wastewater

Product	Fungus	Reference
Single-cell protein (SCP) and soluble protein	*Aspergillus niger*	Hamdi et al., 1991a
Mushroom cultivation	*Pleurotus eryngii* and *P. pulmonarius*	Zervakis et al., 1996
Fertilizer	*Aspergillus niger*	Vassilev et al., 1997
Biogas	*Pleurotus ostreatus*	Fountoulakis et al., 2002
Laccase and manganese peroxidase (MnP)	*Panus tigrinus*	Fenice et al., 2003

An approach to the use of OMW as a fertilizer (Lombardo, 1988) and to formulate a process for OMW-based fertilizer production (Vassilev et al., 1997) has been suggested. Aerobic bioremediation of OMW can produce high-quality compost containing a good supply of nutrients (Tomati et al., 1996). Biomethanation was inhibited during the anaerobic digestion of OMW prefermented with *P. chrysosporium* at a low hydraulic loading rate (Gharsallah et al., 1999). Biogas produced by thermally processed OMW with *P. ostreatus* was higher than that produced by raw and thermally processed OMW, respectively (Fountoulakis et al., 2002). OMW was also used as a growth medium for the production of laccase and MnP from *Panus tigrinus* CBS 577.79 (Fenice et al., 2003). Solid-state fermentation was more suitable than submerged fermentation. The highest levels of laccase and MnP were achieved in a stirred-tank reactor (4600 ± 98 U/l on day 13) and in an airlift reactor (410 ± 22 U/l on day 7), respectively.

2.4   BIOTECHNOLOGY

Microorganisms exist in nature as members of complex and mixed communities. In industrial wastewater bioreactors, microorganisms can be employed as model systems to understand the evolution of new metabolic pathways in natural ecosystems. In general, a wastewater bioreactor is geared to a process that dictates the chemical composition of the waste stream. A unique ecosystem evolves over time to degrade the components of a particular stream. For example, a bioreactor for starch processing will be enriched with starch degraders, whereas the bioreactors of acidogenic effluents will be enriched with acidogenic degraders. Modification of the processes contributes to a change of components of the waste stream that causes the microorganisms in the bioreactor to adapt. This microbial diversity in a bioreactor leads to the development of new metabolic pathways (Bramucci and Nagarajan, 2000).

A wide variety of engineering parameters are regularly monitored during operation of wastewater bioreactors. However, the microorganisms in bioreactors have never been well characterized. Polymerase chain reaction (PCR) amplification of 16S rRNA gene sequences is used to detect the bacterial diversity in ecosystems. Similar approaches have been employed to study the structure and activities of microbial population in wastewater bioreactors (Amann et al., 1998; Oerther et al., 2000). The microbial population in a wastewater bioreactor can be characterized by two approaches: inoculating a variety of cultures with sludge from the bioreactor, and establishment of enriched cultures by inoculating samples of sludge into a minimal salts medium supplemented with a single aromatic compound (e.g., benzene, phenol) as a sole source of carbon and energy. PCR amplification of 16S rRNA is performed on bacterial isolates from standard microbiological media and enrichment cultures. Based on the similarity with 16S rRNA from the GenBank database, the isolates are assigned to groups or species. Bramucci

and Nagarajan (2000) assigned 27 groups or species based on a bacteriai culture from a wastewater bioreactor on standard microbiological media, isolated additional bacteria from enrichment cultures, and tested the metabolic and genetic diversity from enrichment cultures. A phylogenetic tree derived from 16S rRNA gene sequence data for bacteria cultured from an industrial wastewater bioreactor has also been constructed and aligned. Bacteria with novel metabolic pathways are also identified. Use of genomic technology can develop novel biocatalysts useful for industrial wastewater bioreactors. Unfortunately, at present, most work is focused on bacteria rather than fungi.

2.5 CONCLUSIONS AND FUTURE PERSPECTIVES

The use of fungi in wastewater treatment is not new or old. It is correct that there are many types of wastewaters that cannot be degraded by fungi or may be attributed to the slow growth of fungi or the difficulty of using them in bioreactors. With the advent of new manufacturing products, new types of wastewaters will be produced and may be amenable to degradation by fungi. It is also important to identify and characterize fungal communities already present in wastewater bioreactors related to different types of waste streams.

Various types of bioreactors have been established in the treatment of wastewaters by fungi. In certain cases, fermentation models to predict the performance of a system under various operating conditions are known. A wide scope exists for the use of computer models based on different operating parameters. Enzyme treatment has already opened a door for the treatment of wastewaters, but more needs to be accomplished. The use of immobilized fungal cells or enzymes in beads or carriers has already achieved good success and reduced the treatment cost. Wastewater utilization concomitant with the production of value-added products is quite attractive. Several types of wastewater processing industries have installed such processes at their facilities. Nutrient supplementation(s) may be required. Such processes have been patented. New products, including enzymes, are being produced to offset the treatment cost of certain wastewaters.

REFERENCES

Sections 2.1 and 2.2

Barratt, P.A., A. Baumgartl, N. Hannay, M. Vetter, and F. Xiong (1997) Chemox™: advanced wastewater treatment with the impinging zone reactor. *Water Sci. Technol.* **35**: 347–352.

Furusaki, S. (1988) Engineering aspects of immobilized biocatalysts. *J. Chem. Eng. Jpn.* **21**: 219–230.

Godia, F., and C. Sola (1995) Fluidized bed bioreactors. *Biotechnol. Prog.* **11**: 479–497.

Grady, C.P.L., Jr., G.T. Daigger, and H.C. Lim (1999) *Biological Wastewater Treatment*, 2nd ed. Marcel Dekker, New York.

Jianlong, W., S. Hanchang, and Q. Yi (2000) Wastewater treatment in a hybrid biological reactor (HBR): effect of organic loading rates. *Process Biochem.* **36**: 297–303.

Lazarova, V., and J. Manem (1996) An innovative process for wastewater treatment: the circulating floating bed reactor. *Water Sci. Technol.* **34**: 89–99.

Mann, J.G., and Y.A. Liu (1999) *Industrial Water Reuse and Wastewater Minimization*. McGraw-Hill, New York.

Mulder, R., T.L. Vereijken, C.T. Frijters, and S.H. Vellinga (2001) Future perspectives in bioreactor development. *Water Sci. Technol.* **44**: 27–32.

Peters, T.A., R. Gunther, and K. Vossenkaul (2000) Membrane bioreactors in wastewater treatment. *Filtrat. Sep.* **37**: 18–21.

Rossi, G. (2001) The design of bioreactors. *Hydrometallurgy* **59**: 217–231.

Schugerl, K. (1997) Three-phase-biofluidization: application of three-phase fluidization in the biotechnology—a review. *Chem. Eng. Sci.* **52**: 3661–3668.

Schuler, M.L., and F. Kargi (1992) *Bioprocess Engineering: Basic Concepts.* Prentice Hall, Englewood Cliffs, NJ.

Schwuger, M.J., G. Subklew, and N. Woller (2001) New alternatives for wastewater remediation with complexing surfactants. *Colloids Surf. A* **186**: 229–242.

Tanaka, H., E. Nakamura, Y. Minamiyama, and T. Toyoda (1994) BOD biosensor for secondary effluent from wastewater treatment plants. *Water Sci. Technol.* **30**: 215–227.

Visvanathan, C., R.B. Aim, and K. Parameshwaran (2000) Membrane separation bioreactors for wastewater treatment. *Crit. Rev. Environ. Sci. Technol.* **30**: 1–48.

Walters, M.J., and G.K. Robinson (1997) Environmental biotechnology: monitoring, mobilizing and mineralizing pollution. *Tibtech* **15**: 280–282.

Zbontar, L., and P. Glavic (2000) Total site: wastewater minimization, wastewater reuse and regeneration use. *Resour. Conserv. Recycl.* **30**: 261–275.

Section 2.3.1

Chew, T.Y., and Y.L. Shim (1990) *A Survey of Sago Processing Wastes*. Report to the Environmental Biotechnology Section, National Biotechnology Council, MARDI, Kuala Lumpur, Malaysia.

Chigusa, K., and M. Matsumaru (1991) Process for high-lat treatment of carbohydrate-containing waste water. U.S. patent 5,075,008.

Coleman, R. (1990) Biodegradable plastics from potato waste double savings to environment. *Agric. Eng.* **71**: 20–22.

Damasceno, S., M.P. Cereda, G.M. Pastore, and J.G. Oliveira (2003) Production of volatile compounds by *Geotrichum fragrans* using cassava wastewater as substrate. *Process Biochem.* **39**: 411–414.

Fujita, M., K. Iwahori, and K. Yamakawa (1993) Pellet formation of fungi and its application to starch wastewater treatment. *Water Sci. Technol.* **28**: 267–274.

Hang, Y.D. (1980) Assimilation of lemonade-processing wastewater by yeasts. *Appl. Environ. Microbiol.* **39**: 470–472.

Hang, Y.D., D.F. Splittstoesser, and R.L. Landschoot (1973) Use of yeast in the treatment of sauerkraut waste. In: *Food Processing Waste Management*: *Proceedings of the Cornell Agricultural Waste Management Conference*, Mar. 26–28, Syracuse, NY.

Hu, T.L. (1989) Treatment of vermicelli wastewater by an acid-tolerant, starch-degrading yeast. *Biol. Wastes* **28**: 163–174.

Jin, B., H.J. van Leewen, B. Patel, and Q. Yu (1998) Utilization of starch processing wastewater for production of microbial biomass protein and fungal α-amylase by *Aspergillus oryzae. Bioresour. Technol.* **66**: 201–206.

Jin, B., H.J. van Leewen, B. Patel, H.W. Doelle, and Q. Yu (1999a) Production of fungal protein and glucoamylase by *Rhizopus oligosporus* from starch processing wastewater. *Process Biochem.* **34**: 59–65.

Jin, B., H. van Leewen, and B. Patel (1999b) Mycelial morphology and fungal protein production from starch processing wastewater in submerged cultures of *Aspergillus oryzae. Process Biochem.* **34**: 335–340.

Karam, J., and J.A. Nicell (1997) Potential applications of enzymes in waste treatment. *J. Chem. Tech. Biotechnol.* **69**: 141–153.

Karim, M.I.A., and W.A. Sistrunk (1985) The use of selected strains of yeasts in the treatment of processing wastewater from lye-peeled and steam-peeled potatoes. *J. Food Process. Preserv.* **8**: 175–190.

Manilal, V.B., C.S. Narayanan, and C. Balagopalan (1991) Cassava starch effluent treatment with concomitant SCP production. *World J. Microbiol. Biotechnol.* **7**: 185–190.

Manjunath, P., B.C. Shenoy, and M.R. Rao (1983) Review: fungal glucoamylases. *J. Appl. Biochem.* **5**: 235–260.

Markl, H., and G. Reinold (1994) Biogas-Turmreaktor, ein neues Konzept fur die anaerobe Abwasserreinigung. *Chem. Ind. Technik.* **66**: 534–536.

Nandan, R., and S. Raisuddin (1992) Fungal degradation of industrial wastes and wastewater. In: *Handbook of Applied Mycology*, D.K. Arora, B. Rai, K.G. Mukerji, and G. Knudsen, eds. Marcel Dekker, New York, Vol. 4, pp. 931–957.

Nguyen, Q.D., J.M. Rezessy-Szabo, M. Claeyssens, I. Stals, and A. Hoschke (2002) Purification and characterization of amylolytic enzymes from thermophilic fungus *Thermomyces lanuginosus* strain ATCC 34626. *Enzyme Microb. Technol.* **31**: 345–352.

Norouzian, D., A. Akbarzadeh, J.M. Scharer, and M.M Young (2006) Fungal glucoamylases. *Biotechnol. Adv.* **24**: 80–85.

Pasari, A.B., R.A. Korus, and R.C. Heimsch (1989) A model for continuous fermentations with amylolytic yeasts. *Biotechnol. Bioeng.* **33**: 338–343.

Reinold, G., S. Merrath, F. Lennemann, and H. Markl (1996) Modelling the hydrodynamics and the liquid-mixing behaviour of a biogas tower reactor. *Chem. Eng. Sci.* **51**: 4065–4073.

Shim, Y.L. (1992) Utilization of sago hampas by microfungi. M. Biotechnol. thesis, University of Malaya, Kuala Lumpur, Malaysia.

Shoemaker, S. (1986) The use of enzymes for waste management in the food industry. In: *Biotechnology in Food Processing*, S.K. Harlander and T.P. Labuza, eds. Noyes Publications, Park Ridge, NJ, pp. 259–267.

Stevenson, K.E., D.E. Black, and R.N. Costilow (1979) Aerobic fermentations of pickle process brine by *Candida utilis*. *J. Food Sci.* **44**: 181–185.

Thanh, V.N., M.S. Smit, N. Moleleki, and J.W. Fell (2004) *Rhodotorula cycloclastica* sp. nov., *Rhodotorula retinophila* sp. nov., and *Rhodotorula terpenoidalis* sp. nov., three limonene-utilizing yeasts isolated from soil. *FEMS Yeast Res.* **4**: 857–863.

Vikineswary, S., and P. Nadaraj (1992) Fermentation of sago processing residue hampas by submerged fungal cultures. In: *Proceedings of the National Symposium on Fermentation Technology*, MARDI, Kuala Lumpur, Malaysia, pp. 118–121.

Vikineswary, S., Y.L. Shim, J.J. Thambirajah, and N. Blakebrough (1994) Possible microbial utilization of sago processing wastes. *Resour. Conserv. Recycl.* **11**: 289–296.

Zhang, Y., B.E. Rittmann, J. Wang, Y. Sheng, J. Yu, H. Shi, and Y. Qian (2005) High carbohydrate wastewater treatment by IAL-CHS with immobilized *Candida tropicalis*. *Process Biochem*. **40**: 857–863.

Section 2.3.2

Adam, A.C., J.A. Prieto, M. Rubio-Texeira, and J. Polaina (1999) Construction of a lactose-assimilating strain of baker's yeast. *Yeast* **15**: 1299–1305.

Bainotti, A.F., J.C. Basilico, and M.S. Carrasco de Mendoza (1987) Optimizing conditions for the discontinuous production of unicellular protein using whey. *Rev. Argent. Microbiol.* **19**: 1–7.

Bales, S.A., and F.J. Castillo (1979) Production of lactase by *Candida pseudotropicalis* grown in whey. *Appl. Environ. Microbiol.* **37**: 1201–1205.

Banerjee, M., A. Chakrabarty, and S.K. Majumdar (1982) Immobilization of yeast cells containing β-galactosidase. *Biotechnol. Bioeng.* **24**: 1839–1850.

Barnett, J.A. (1981) The utilization of disaccharides and some other sugars by yeasts. In: *Advances in Carbohydrate Chemistry and Biochemistry*, R.S. Tipson and D. Horton, eds. Academic Press, New York, Vol. 38, pp. 347–404.

Barnett, J.A., R.W. Payne, and D. Yarrow (1979) *A Guide to Identifying and Classifying Yeasts*. Cambridge University Press, Cambridge.

Becerra, M., B. Baroli, A.M. Fadda, J.B. Mendez, and M.I.G. Siso (2001) Lactose bioconversion by calcium-alginate immobilization of *Kluyveromyces lactis* cells. *Enzyme Microb. Technol.* **29**: 506–512.

Ben-Hassan, R.M., and A.E. Ghaly (1994) Continuous propagation of *Kluyveromyces fragilis* in cheese for pollution potential reduction. *Appl. Biochem. Biotechnol.* **47**: 89–105.

Berruga, M.I., A. Jaspe, and C. SanJose (1997) Selection of yeast strains for lactose hydrolysis in dairy effluents. *Int. Biodeterior. Biodegrad*. **40**: 119–123.

Blasheck, H.P. (1992) Approaches to making the food processing industry more environmentally friendly. *Trends Food Sci. Technol.* **3**: 107–110.

Carlotti, A., F. Jacob, J. Perrier, and S. Poncet (1991) Yeast production from crude sweet whey by a mixed culture of *Candida kefyr* LY496 and *Candida valida* LY497. *Biotechnol. Lett.* **13**: 437–440.

Castillo, F.J. (1990) Lactose metabolism by yeasts. In: *Yeast: Biotechnology and Biocatalysis*, H. Verachtert and R. De Mot, eds. Marcel Dekker, New York, pp. 297–320.

Cristiani-Urbina, E., N. Ruiz-Ordaz, and J. Galinez-Mayer (1997) Differences in the growth kinetic behaviour of *Torulopsis cremoris* in batch and continuous cultures. *Biotechnol. Appl. Biochem.* **26**: 189–194.

Cristiani-Urbina, E., A.R. Netzahuatl-Munoz, F.J. Manriquez-Rojas, C. Juarez-Ramirez, N. Ruiz-Ordaz, and J. Galindez-Mayer (2000) Batch and fed-batch cultures for the treatment of whey with mixed yeast cultures. *Process Biochem.* **35**: 649–657.

Fenton, D.M. (1982) Solvent treatment for β-D-galactosidase release from yeast cells. *Enzyme Microb. Technol.* **4**: 229–232.

Ghaly, A.E., R.M. Ben-Hassan, and N. Ben-Abdallah (1992) Measurement of heat generated by mixing during the batch production of single cell protein from cheese whey. *Biotechnol. Prog.* **8**: 404–409.

Gomez, A., and F.J. Castillo (1983) Production of biomass and β-D-galactosidase by *Candida pseudotropicalis* grown in continuous culture on whey. *Biotechnol. Bioeng.* **25**: 1341–1357.

Goncalves, J.A., and F.J. Castillo (1981) Partial purification and characterization of β-galactosidase from *Kluyveromyces marxianus*. *J. Dairy Sci.* **65**: 2088–2094.

Gonzalez, J.B., and D.R. Berry (1982) Induction of β-galactosidase in the yeast *Kluyveromyces lactis. Biotechnol. Lett.* **4**: 369–374.

Gonzalez Siso, M.I., E. Ramil, M.E. Cerdan, and M.A Freire (1996) Respirofermentative metabolism in *Kluyveromyces lactis*: ethanol production and the Crabtree effect. *Enzyme Microb. Technol.* **18**: 585–591.

Itoh, T., M. Suzuki, and S. Adachi (1982) Production and characterization of β-galactosidase from lactose-fermenting yeasts. *Agric. Biol. Chem.* **46**: 899–904.

Jelen, P. (1992) Whey: composition, properties, processing and uses. In: *Encyclopaedia of Food Science and Technology*, T.H. Hui, ed. Wiley, New York, Vol. 4, pp. 2835–2845.

Kilara, A., and M.T. Patel (1992) Whey and lactose fermentation. In: *Whey and Lactose Processing*, J.G. Zadow, ed. Elsevier, London, pp. 409–448.

Kumar, V., S. Ramakrishnan, T. Teeri, J.K.C. Knowles, and B.S. Hartley (1992) *Saccharomyces cerevisiae* cells secreting an *Aspergillus niger* β-galactosidase grow on whey permeate. *Biotechnology* **10**: 82–85.

Litchfield, J.H. (1983) Single-cell proteins. *Science* **219**: 740–746.

Maiorella, B.L., and F.J. Castillo (1984) Ethanol, biomass, and enzyme production for whey waste abatement. *Process Biochem.* **19**: 157–161.

Marwaha, S.S., and J.F. Kennedy (1988) Review: Whey: pollution problem and potential utilization. *Int. J. Food Sci. Technol.* **23**: 323–336.

Marwaha, S.S., J.F. Kennedy, H.K. Tewari, and A. Redhu (1989) Characterization and treatment of dairy effluents by free and immobilized yeast. *Process Biochem.* **24**: 46–51.

Moresi, M., M. Patete, and A. Trunfio (1989) Scaling-up of a batch whey fermentation by *K. fragilis. Appl. Microbiol. Biotechnol.* **31**: 495–501.

Moresi, M., A. Trunfio, and E. Parente (1990) Kinetics of continuous whey fermentation by *Kluyveromyces fragilis. J. Chem. Tech. Biotechnol.* **49**: 205–222.

Moulin, G., B. Malige, and P. Galzy (1983) Balanced flora of an industrial fermenter: production of yeast growth from whey. *J. Dairy Sci.* **66**: 21–28.

Perro, D., E. Martegani, B.M. Ranzi, and L. Alberghina (1992) Lactose/whey utilization and ethanol production by transformed *Saccharomyces cerevisiae* cells. *Biotechnol. Bioeng.* **39**: 799–805.

Pigache, S., P. Dhoms, and G. Trystram (1992) Oxygen transfer modeling and simulations for an industrial continuous airlift fermentor. *Biotechnol. Bioeng.* **39**: 923–931.

Ramakrishnan, S., and B.S. Hartley (1993) Fermentation of lactose by yeast cells secreting recombinant fungal lactase. *Appl. Environ. Microbiol.* **59**: 4230–4235.

Rubio-Texeira, M., J.I. Castrillo, A.C. Adam, U.O. Ugalde, and J. Polaina (1998) Highly efficient assimilation of lactose by a metabolically engineered strain of *Saccharomyces cerevisiae. Yeast* **14**: 827–837.

Rubio-Texeira, M., M. Arevalo-Rodriguez, J.L. Lequerica, and J. Polaina (2000) Lactose utilization by *Saccharomyces cerevisiae* strains expressing *Kluyveromyces lactis* LAC genes. *J. Biotechnol.* **84**: 97–106.

Shoemaker, S. (1986) The use of enzymes for waste management in the food industry. In: *Biotechnology in Food Processing*, S.K. Harlander and T.P. Labuza, eds. Noyes Publications, Park Ridge, NJ, pp. 259–267.

Sonawat, H.M., A. Agrawal, and S.M. Dutta (1981) Production of β-galactosidase from *Kluyveromyces fragilis* grown on whey. *Folia Microbiol.* **26**: 370–376.

Sreekrishna, K., and R.C. Dickson (1985) Construction of strains *of Saccharomyces cerevisiae* that grow on lactose. *Proc. Natl. Acad. Sci. USA* **82**: 7909–7913.

Zafar, S., and M. Owais (2005) Ethanol production from crude whey by *Kluyveromyces marxianus. Biochem. Eng. J.* **27**: 295–298.

Section 2.3.3

Alvarino, C.R., M.D.C.E. Llorens, and M.C. Arias (1997) Treatment of pharmaceutical formulation wastes. *Int. Biodeterior. Biodegrad.* **37**: 118.

Halling-Sorensen, B., S. Nors Nielson, P.F. Lanzky, F. Ingerslev, H.C. Holten Lutzhoft, and S.E. Jorgensen (1998) Occurrence, fate and effects of pharmaceutical substances in the environment: a review. *Chemosphere* **36**: 357–393.

Rosen, M., T. Welander, A. Lofqvist, and J. Holmgren (1998) Development of a new process for treatment of a pharmaceutical wastewater. *Water Sci. Technol.* **37**: 251–258.

Zabranska, J., P. Jenicek, and M. Dohanyos (1994) Determination of anaerobic biodegradability of pharmaceutical wastes by methanogenic activity tests. *Water Sci. Technol.* **30**: 103–107.

Section 2.3.4

Burkholder, L., P.R. Burkholder, A. Chu, N. Kostyk, and O.A. Roels (1968) Fish fermentation. *Food Technol.* **22**: 1278–1284.

Grant, R.A. (1976) Protein recovery from meat, poultry and fish processing plants. In: *Food from Waste*, G.G. Birch, K.J. Parker, and J.T. Worgan, eds. Applied Science Publishers, London, pp. 205–220.

Green, J.H., S.L. Paskell, and D. Goldmintz (1976) Lipolytic fermentation of stickwater by *Geotrichum candidum* and *Candida lipolytica*. *Appl. Environ. Microbiol.* **31**: 569–575.

Hottinger, H.H., T. Richardson, C.H. Amundsen, and D.A. Stuiber (1974) Utilization of fish oil by *Candida lipolytica*. II. Optimization of conditions. *J. Milk Food Technol.* **37**: 522–528.

Rydin, S., G. Molin, and I. Nilsson (1990) Conversion of fat into yeast biomass in protein-containing waste-water. *Appl. Microbiol. Biotechnol.* **33**: 473–476.

Tan, K.H., and C.O. Gill (1984) Effect of culture conditions on batch growth of *Saccharomycopsis lipolytica* on olive oil. *Appl. Microbiol. Biotechnol.* **20**: 201–206.

Tan, K.H., and C.O. Gill (1985) Batch growth of *Saccharomycopsis lipolytica* on animal fats. *Appl. Microbiol. Biotechnol.* **21**: 292–298.

Section 2.3.5

Barker, T.W., and J.T. Morgan (1981) The utilization of palm oil processing effluents as substrates for microbial protein production by the fungus *Aspergillus oryzae*. *Eur. J. Appl. Microb. Biotechnol.* **11**: 234–240.

Chan, K.W., T.S. Ping, and R.M Aminuddin (1983) POME utilisation and its future research directions in the palm industry. In: *Proceedings of the Seminar on Land Application of Palm Oil and Rubber Factories Effluents*, Aug. 15–16, Kuala Lumpur, Malaysia, L.K. Huan, A.T. Bachik, and P.Y. Chin, eds. Malaysian Society of Soil Science, Kuala Lumpur, Malaysia, pp. 23–45.

Chen, J., T. Ishi, K. Kirimura, and S. Usami (1990) Isolation of a Deuteromycotinous yeast producing lipase. *Rep. Waseda Univ.* **130**: 33–38 (in Japanese).

Chigusa, K., N. Nishi, M. Matsumaru, and Y. Minegishi (1989) Treatment of food manufacturing wastewater by yeast. *J. Environ. Pollut. Control.* **25**: 1557–1563 (in Japanese).

Chigusa, K., T. Hasegawa, N. Yamamoto, and Y. Watanabe (1996) Treatment of wastewater from oil manufacturing plant by yeasts. *Water Sci. Technol.* **34**: 51–58.

Harada, I. (1986) *Knowledge of Oil and Chemistry: Glyceride*. Miyuki Shobo Co., Tokyo, pp. 36–41 (in Japanese).

Ibrahim, A., B.G. Yeoh, S.C. Cheah, A.N. Ma, et al. (1984) Thermophilic anaerobic contact digestion of palm oil mill effluent. *Water Sci. Technol.* **17**: 155–166.

Japan Oil Chemists Society (1971) *Standard Methods for the Analysis of Oils, Fats and Derivatives: Fatty Acids*. Japan 4.4.1. JOCS, Tokyo.

Karim, M.I.A., and A.Q.A. Kamil (1989) Biological treatment of palm oil mill effluent using *Trichoderma viride*. *Biol. Wastes* **27**: 143–152.

Kou, J.S., T. Kodama, and T. Minoda (1983) Screening of yeasts and cultural conditions for cell production from palm oil. *Agric. Biol. Chem.* **47**: 1207–1212 (in Japanese).

Montet, D., R. Ratomahenina, A. Ba, M. Pina, J. Graille, and P. Galzy (1983) Production of single cell protein from vegetable oils. *J. Ferment. Technol.* **61**: 417–420.

Nawamura, Y., T. Nishi, and K. Chigusa (1992) Process for high load treatment of wastewater containing fish meal by yeast. *J. Operat. Install.* **11**: 99–104 (in Japanese).

Oswal, N., P.M. Sarma, S.S. Zinjarde, and A. Pant (2002) Palm oil mill effluent treatment by a tropical marine yeast. *Bioresour. Technol.* **85**: 35–37.

Sinnappa, S. (1979) Treatment studies on palm oil mill waste effluent. In: *Proceedings of the International Conference on Water Pollution Control in Developing Countries*, Asian Institute of Technology, Bangkok, Thailand, pp. 525–537.

Suwandi, M.S., and A.A. Mohd (1984) Growth of *Penicillium chrysogenum* in palm oil mill effluent concentrate. In: *Proceedings of the Regional Seminar-Workshop on Biotechnology in Industrial Developments*, Mar. 27–30, Universiti Pertanian Malaysia, Serdang, Selangor, Malaysia.

Tosaki, T., H. Ishikawa, and K. Chigusa (1991) Wastewater management of food industrial processing by yeasts. In: *Proceedings of the 28th Annual Conference of Sewage Treatment*, pp. 752–754 (in Japanese).

Section 2.3.6

Arnold, J.L., J.S. Knapp, and C.L. Johnson (2000) The use of yeasts to reduce the polluting potential of silage effluent. *Water Res.* **34**: 3699–3708.

Beck, L. (1989) A review of farm waste pollution. *J. Inst. Water Environ. Manag.* **3**: 467–477.

Deans, E.A., and I.F. Svoboda (1992) Aerobic treatment of silage effluent-laboratory experiments. *Bioresour. Technol.* **40**: 23–27.

Dunlea, A.P., and V.A. Dodd (1989) The application of membrane filtration to silage effluent. *Can. Agric. Eng.* **31**: 39–43.

Galanos, E., K.R. Gray, A.J. Biddlestone, and K. Thayanithy (1995) The aerobic treatment of silage effluent: effluent characterization and fermentation. *J. Agric. Eng. Res.* **62**: 271–279.

Haigh, P.M. (1994) A review of agronomic factors influencing grass silage effluent production in England and Wales. *J. Agric. Eng. Res.* **57**: 73–87.

Ministry of Agriculture, Fisheries and Food (1999) Surveys of agriculture: 1st December 1998, UK. *MAFF Statistics News Release, Stats. 37/99.* Government Statistical Service (C and S Branch), York, UK.

National Rivers Authority (1992) The influence of agriculture on the quality of national water in England and Wales. *Report by the National Rivers Authority.* National Rivers Authority, Croydon, UK, 153 pp.

O'Brien, M., P. O'Kiely, P.D. Forristal, and H.B. Fuller (2005) Fungi isolated from contaminated baled grass silage on farms in the Irish midlands. *FEMS Microbiol. Lett.* **247**: 131–135.

Olsen, R.A., and T.A. Pedersen (1974) Growth of *Torulopsis glabrata* in effluents of grass silage. *Acta Agric. Scand.* **24**: 160–167.

Patterson, D.C., and D.J. Kilpatrick (1991) Effluent from grass silage for finishing pigs. *J. Agric. Sci.* **116**: 119–124.

Skaar, I., and H. Stenwig (1996) Malt-yeast extract-sucrose agar, a suitable medium for enumeration and isolation of fungi from silage. *Appl. Environ. Microbiol.* **62**: 3614–3619.

Snerik, E., A. Bergheim, A.R. Selmer-Olden, and A. Sivertsen (1977) Investigations of five water courses in Jacren-region polluted with silage juice 1971 to 76-1, 1971–73. *Sci. Rep. Agric. Univ. Norway* **56**: 1–32.

UK Environment Agency (1996) *Water Pollution Incidents in England and Wales, 1995*. Environment Agency, Bristol, UK.

UK Environment Agency (1997) *Water Pollution Incidents in England and Wales, 1996*. Environment Agency, Bristol, UK.

UK Environment Agency (1998) *Water Pollution Incidents in England and Wales, 1997*. Environment Agency, Bristol, UK.

UK Environment Agency (1999) *Water Pollution Incidents in England and Wales, 1998*. Environment Agency, Bristol, UK.

Section 2.3.7

Cama, F.J., and V.H.J. Edwards (1970) Single-cell protein from chemical wastes: the growth of *Candida utilis* on sodium acetate. *Ferment. Technol.* **48**: 787–794.

Defrance, M.B. (1993) Étude d'un racteur levurien pour le traitement d'effluents d'industries alimentaires. Ph.D. dissertation, Université Montpellier II, Montpellier, France.

Dinoupoulou, G., T. Rudd, and J.N. Lester (1988) Anaerobic acidogenesis of a complex wastewater. *Biotechnol. Bioeng.* **31**: 958–968.

Edeline, F. (1993) *L'Epuration biologique des eaux*. Technique et Documentation, Paris.

Elmaleh, S., M.B. Defrance, C. Ghommidh, and J.M. Navarro (1995) Racteur levurien pour l'epuration d'effluents concentres des industries alimentaires. *Recent Prog. Genie Procedes* **9**: 7–12.

Elmaleh, S., M.B. Defrance, C. Ghommidh, and J.M. Navarro (1996) Acidogenic effluents treatment in a yeast reactor. *Water Res.* **30**: 2526–2529.

Elmaleh, S., M.B. Defrance, and C. Ghommidh (1999) Organic acids oxidation by *Candida utilis*: application to industrial waste water treatment. *Process Biochem.* **35**: 441–449.

Herrero, A.A. (1983) End-product inhibition in anaerobic fermentations. *Trends Biotechnol.* **1**: 49–53.

Jackson, J.V., and V.H.J. Edwards (1975) Kinetics of substrate inhibition of exponential yeast growth. *Biotechnol. Bioeng.* **17**: 943–964.

Ko, R.Y.C., and V.H.J. Edwards (1975) Transient kinetics of growth in batch and continuous culture with in a inhibitory carbon and energy source. *Biotechnol. Bioeng.* **16**: 965–983.

Laulan, P.H. (1987) Traitement biologique d'effluents toxiques des industries agro-alimentaires. Ph.D. dissertation, École Centrale des Arts et Manufactures, Paris.

Lawford, H.G., and J.D. Rousseau (1993) Effects of pH and acetic acid on glucose and xylose metabolism by a genetically engineered ethanologenic *Escherichia coli*. *Appl. Biochem. Biotechnol*. **39**: 301–322.

Maugeri, F.F. (1980) Étude d'une nouvelle voie de production de biomasse micro-bienne a partir de cellulose, Thèse de Docteur-Ingenieur 41, INSA, Toulouse, France.

Maugeri, F.F., and G. Goma (1980) SCP production from organic acids with *Candida utilis. Rev. Microbiol*. **19**: 446–454.

Ortiz, C.P., J.-P. Steyer, and A. Bories (1997) Carbon and nitrogen removal from wastewater by *Candida utilis*: kinetic aspects and mathematical modelling. *Process Biochem*. **32**: 179–189.

Section 2.3.8

Aggelis, G., C. Ehaliotis, F. Nerud, I. Stoychev, G. Lyberatos, and G.I. Zervakis (2002) Evaluation of white-rot fungi for detoxification and decolorization of effluents from the green olive debittering process. *Appl. Microbiol. Biotechnol*. **59**: 353–360.

Balice, V., C. Carieri, O. Cera, and B. Rindane (1988) The fate of tannin-like com-pounds from olive mill effluents in biological treatments. In: *Proceedings of the Fifth International Symposium on Anaerobic Digestion*, E.R. Hall and P.N. Hobson, eds. Pergamon Press, Oxford, pp. 275–280.

Blanquez, P., G. Caminal, M. Sarra, M.T. Vicent, and X. Gabarrell (2002) Olive oil mill waste waters decolorization and detoxification by the white-rot fungus *Phanerochaete flavido-alba. Biotechnol. Prog*. **18**: 660–662.

Borja, R., A. Martin, R. Maestro, J. Alba, and J.A. Fiestas (1992) Enhancement of the anaerobic digestion of olive mill wastewater by the removal of phenolic inhibi-tors. *Process Biochem*. **27**: 231–237.

Borja, R., S.E. Garrido, L. Martinez, A. Ramos-Cormenzana, and A. Martin (1993) Kinetic study of anaerobic digestion of olive mill wastewater previously fermented with *Aspergillus terreus. Process Biochem*. **28**: 397–404.

Borja, R., A. Martin, V. Alonso, I. Garcia, and C.J. Banks (1995) Influence of differ-ent aerobic pretreatments on the kinetics of anaerobic digestion of olive mill wastewater. *Water Res*. **29**: 489–495.

Borja, R., J. Alba, and C.J. Banks (1997) Impact of the main phenolic compounds of olive mill wastewater (OMW) on the kinetic acetoclastic methanogenesis. *Process Biochem*. **32**: 121–133.

Capasso, R., A. Evidente, L. Schivo, G. Orru, M. Marcialis, and G. Cristinzio (1995) Antibacterial polyphenols from olive mill waste waters. *J. Appl. Bacteriol*. **7**: 393–398.

D'Annibale, A., S.R. Stazi, V. Vinciguerra, E. Di Mattia, and G.G. Sermanni (1999) Characterization of immobilized laccase from *Lentinula edodes* and its use in olive-mill wastewater treatment. *Process Biochem*. **34**: 697–706.

D'Annibale, A., S.R. Stazi, V. Vinciguerra, and G.G. Sermanni (2000) Oxirane-immobilized *Lentinula edodes* laccase: stability and phenolics removal efficiency in olive mill wastewater. *J. Biotechnol*. **77**: 265–273.

D'Annibale, A., R. Casa, F. Pieruccetti, M. Ricci, and R. Marabottini (2004a) *Lentinus edodes* removes phenols from olive-mill wastewater: impact on durum wheat (*Triticum durum* Desf.) germinability. *Chemosphere* **54**: 887–894.

D'Annibale, A., M. Ricci, D. Quarantino, F. Federici, and M. Fenice (2004b) *Panus tigrinus* efficiently removes phenols, color and organic load from olive-mill wastewater. *Res. Microbiol.* **155**: 596–603.

Duran, N., and E. Esposito (2000) Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. *Appl. Catal. B* **28**: 83–99.

Ettayebi, K., F. Errachidi, L. Jamai, M.A. Tahri-Jouti, K. Sendide, and M. Ettayebi (2003) Biodegradation of polyphenols with immobilized *Candida tropicalis* under metabolic induction. *FEMS Microbiol. Lett.* **223**: 215–219.

Fenice, M., G.G. Semanni, F. Federici, and A. D'Annibale (2003) Submerged and solid-state production of laccase and Mn-peroxidase by *Panus tigrinus* on olive mill wastewater-based media. *J. Biotechnol.* **100**: 77–85.

Fountoulakis, M.S., S.N. Dokianakis, M.E. Kornaros, G.G. Aggelis, and G. Lyberatos (2002) Removal of phenolics in olive mill wastewaters using the white-rot fungus *Pleurotus ostreatus*. *Water Res.* **36**: 4735–4744.

Garcia, I.G., P.R.J. Pena, J.L.B. Venceslada, A.M. Martin, M.A.M. Santos, and E.R. Gomez (2000) Removal of phenol compounds from olive mill wastewater using *Phanerochaete chrysosporium*, *Aspergillus niger*, *Aspergillus terreus* and *Geotrichum candidum*. *Process Biochem.* **35**: 751–758.

Gharsallah, N., M. Labat, F. Aloui, and S. Sayadi (1999) The effect of *Phanerochaete chrysosporium* pretreatment of olive mill waste waters on anaerobic digestion. *Resour. Conserv. Recycl.* **27**: 187–192.

Hamdi, M., A. Khadir, and J.L. Garcia (1991a) The use of *Aspergillus niger* for the bioconversion of olive mill waste-waters. *Appl. Microbiol. Biotechnol.* **34**: 828–831.

Hamdi, M., H. BouHamed, and R. Ellouz (1991b) Optimization of the fermentation of olive mill waste-waters by *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* **36**: 285–288.

Israilides, C.J., A.G. Vlyssides, V.N. Mourafeti, and G. Karvouni (1997) Olive oil wastewater treatment with the use of an electrolysis system. *Bioresour. Technol.* **61**: 163–170.

Jaouani, A., S. Sayadi, M. Vanthournhout, and M.J. Penninckx (2003) Potent fungi for decolourisation of olive oil mill wastewaters. *Enzyme Microb. Technol.* **33**: 802–809.

Kissi, M., M. Mountadar, O. Assobhei, E. Gargiulo, G. Palmieri, P. Giardina, and G. Sannia (2001) Roles of two white-rot basidiomycete fungi in decolorisation and detoxification of olive mill wastewater. *Appl. Microbiol. Biotechnol.* **57**: 221–226.

Kyriacou, A., K.E. Lasaridi, M. Kotsou, A. Balis, and G. Pilidis (2005) Combined bioremediation and advanced oxidation of green table olive processing wastewater. *Process Biochem.* **40**: 1401–1408.

Linares, A., J.M. Caba, F. Ligero, T. de la Rubia, and J. Martinez (2003) Detoxification of semisolid olive-mill wastes and pine-chip mixtures using *Phanerochaete flavido-alba*. *Chemosphere* **51**: 887–891.

Lombardo, N. (1988) Cultivation of the olive: a typical tree of the Mediterrean basin. *Outlook Agric.* **17**: 183–185.

Martin, A., R. Borja, I. Garcia, and J.A. Fiestas (1991) Kinetics of methane production from olive mill wastewater. *Process Biochem.* **26**: 101–107.

Martirani, L., P. Giardina, L. Marzullo, and G. Sannia (1996) Reduction of phenol content and toxicity in olive oil mill waste waters with the ligninolytic fungus *Pleurotus ostreatus. Water Res.* **30**: 1914–1918.

Millan, B., R. Lucas, A. Robles, T. Garcia, G.A. de Cienfuegos, and A. Galvez (2000) A study on the microbiota from olive-mill wastewater (OMW) disposal lagoons, with emphasis on filamentous fungi and their biodegradative potential. *Microbiol. Res.* **155**: 143–147.

Moreno, E., J. Perez, A. Ramos-Cormenzana, and J. Martinez (1987) Antimicrobial effect of wastewater from olive oil extraction plants selecting soil bacteria after incubation with diluted waste. *Microbios* **51**: 169–174.

Robles, A., R. Lucas, G.A. de Cienfuegos, and A. Galvez (2000) Biomass production and detoxification of wastewaters from the olive oil industry by strains of *Penicillium* isolated from wastewater disposal ponds. *Bioresour. Technol.* **74**: 217–221.

Sainz-Jimenez, C., and G. Gomez-Alarcon (1986) Effects of vegetational water on fungal microflora. In: *Proceedings of the International Symposium on Olive Byproducts Valorization.* Food and Agriculture Organization, Madrid, Spain, pp. 61–76.

Sanjust, E., R. Pompei, A. Rescigno, A. Rinaldi, and M. Ballero (1991) Olive milling wastewater as a medium for growth of four *Pleurotus* species. *Appl. Biochem. Biotechnol.* **31**: 223–235.

Sayadi, S., and R. Ellouz (1992) Decolorization of olive mill waste-water by the white-rot fungus *Phanerochaete chrysosporium*: involvement of the lignin degrading system. *Appl. Microbiol. Biotechnol.* **37**: 813–817.

Sayadi, S., and R. Ellouz (1995) Roles of lignin peroxidase and manganese peroxidase from *Phanerochaete chrysosporium* in the decolorization of olive mill wastewaters. *Appl. Environ. Microbiol.* **61**: 1098–1103.

Sayadi, S., F. Zorgani, and R. Ellouz (1996) Decolorization of olive mill waste-waters by free and immobilized *Phanerochaete chrysosporium* cultures. *Appl. Biochem. Biotechnol.* **56**: 265–276.

Tomati, U., E. Galli, F. Fiorelli, and L. Pasetti (1996) Fertilizers from composting of olive-mill wastewaters. *Int. Biodeterior. Biodegrad.* **38**: 155–162.

Vassilev, N., M. Fenice, and F. Federici (1996) Rock phosphate solubilization with gluconic acid produced by *Penicillium variabile* P16. *Biotechnol. Tech.* **10**: 585–588.

Vassilev, N., M. Fenice, F. Federici, and R. Azcon (1997) Olive mill waste water treatment by immobilized cells of *Aspergillus niger* and its enrichment with soluble phosphate. *Process Biochem.* **32**: 617–620.

Vinciguerra, V., A. D'Annibale, G. Delle Monache, and G.G. Sermanni (1995) Correlated effects during the bioconversion of waste olive waters by *Lentinus edodes. Bioresour. Technol.* **51**: 221–226.

Vitolo, S., L. Petarca, and B. Bresci (1999) Treatment of olive oil industry wastes. *Bioresour. Technol.* **67**: 129–137.

Zervakis, G., P. Yiatras, and C. Balis (1996) Edible mushrooms from olive oil mill wastes. *Int. Biodeterior. Biodegrad*. **38**: 237–243.

Section 2.4

Amann, R., et al. (1998) Monitoring the community structure of wastewater treatment plants: a comparison of old and new techniques. *FEMS Microbiol. Ecol.* **25**: 205–215.

Bramucci, M.G., and V. Nagarajan (2000) Industrial wastewater bioreactors: sources of novel microorganisms for biotechnology. *Tibtech* **18**: 501–505.

Oerther, D.B., et al. (2000) Monitoring precursor 16S rRNAs of *Acinetobacter* spp. in activated sludge wastewater treatment systems. *Appl. Environ. Microbiol.* **66**: 2154–2165.

3

FUNGAL TREATMENT OF DISTILLERY AND BREWERY WASTES

3.1 INTRODUCTION

Distillery wastewater is produced as a result of distillation of ethanol produced in the fermentation of carbohydrates, which pose considerable problems in disposal or treatment. Various types of raw materials, such as sugarcane, beet, grains, and fruit, are being employed for the production of ethanol in these industries. The alcohol can be produced from four types of raw materials (e.g., sugar crops, starch crops, dairy products, and cellulose materials). For every liter of ethanol produced, up to 20 L of stillage is generated (Guruswami, 1988; Algur and Gokalp, 1991; van Haandel and Catunda, 1994). In a typical distillery, over a half million liters of stillage is generated every day. Lele et al. (1989) calculated the pollution level of stillage as being equivalent to the sewage of a city with a population of 500 000.

The most easily fermentable raw materials are derived from sugar-based crops such as sugar beet and sugarcane molasses. However, the sugar industry produces several grades of blackstrap molasses, depending on the sugar and ash contents and color. In 1995 there were 250 distilleries in India, producing 1.1 billion liters of ethanol from sugarcane molasses (Singh and Nigam, 1995). In Brazil, the production of ethanol was 16.2 billion liters in 1997, about 79% produced from sugarcane juice and 21% from molasses by-product (DNDE, 1998). In the United States, 57 facilities produced an estimated 6.9 billion liters of ethanol in 1999 (Wilkie et al., 2000). In Japan, the output of *Shochu*

spirit is 0.2 billion liters, with the production of distillery wastewater about 3×10^8 L since 1986 (Kida et al., 1995). However, the production of stillage varies depending on the raw materials and ethanol production processes.

Molasses spent wash (MSW) acts as a water pollutant in many ways. One of the important features of molasses spent wash is its dark brown color. The color components disrupt the flow of penetration of sun rays in surface waters, which in turn reduces the photosynthetic activity and is detrimental to aquatic life. The high COD load results in eutrophication of contaminated waterways. Such discharge can also increase the temperature of the receiving waters, thus reducing the amount of dissolved oxygen content. In addition, an acidic effluent decreases the pH by dissolving some metals in flowing water. Disposal on land is equally hazardous, as it reduces the soil alkalinity and manganese availability, inhibits seed germination, and destroys crops and vegetation.

Little is known as to the final disposition of stillage and treated stillage effluents. In Brazil, methods have been developed for the utilization of stillage nutrients and land application of untreated stillage on sugarcane fields (de Menezes, 1989). Treatment options are being considered for decolorization, odor control, and nutrient removal prior to acceptable discharge into surface water or any other water body. The long-term impact on the agronomic properties of soils of discharge of treated or untreated stillage effluents requires further study.

3.2   COMPOSITION AND CHARACTERISTICS OF STILLAGE

Molasses stillage has a high ash content, low pH, high concentration of mineral salts, and a BOD range of 45 to 65 g/dm^3 (Algur and Gokalp, 1991). The high BOD value is attributed to the use of 90% or more of the organic, nonfermentable raw material that ends up as waste from the process. *Shochu* distillery wastewater contains suspended solids of 40000 to 50000 mg/l and a high BOD concentration of 40000 to 80000 mg/l (Kida et al., 1995). The composition of sugar beet and sugarcane molasses stillage includes various organic compounds, including acetic acid, lactic acid, glycerol, and reducing sugars. The calcium, magnesium, and *ortho*-phosphate contents in sugarcane stillage are much higher than in sugar beet stillage. The COD/organic dry matter ratio in sugarcane stillage is 1.3 times greater than in sugar beet stillage (Baerwald and Lee, 1989). FitzGibbon et al. (1995) showed spent wash as a recalcitrant waste with a high COD content of 85 170 mg/l, containing inhibitory phenolic (gallic and vanillic acids) compounds. The MSW is recalcitrant, owing to the presence of melanoidins, and contributes color to the effluent. These compounds show antioxidant properties, are inhibitory to fermentation, and are toxic to many microorganisms present in wastewater treatment processes. An average composition of sugarcane molasses distillery effluent and sugarcane molasses spent wash from India has been described

(Singh and Nigam, 1995; Raghukumar and Rivonkar, 2001). The composition of stillages originating from molasses (cane, blackstrap, and beet), grain, malt, cassava, sulfite waste liquor, wine, and fruit has been profiled by Friedrich et al. (1992). Beet molasses has a COD of 80.5 g/l, Kjeldahl-N of 1.8 g/l, soluble phosphorus of 0.12 g/l, sulfates of 5 g/l, and total phenols of 0.450 g/l (Jimenez et al., 2003).

Fermentation of molasses with yeasts and refining the alcohol produced from the fermentation broth by distillation is a common agroindustrial activity throughout the world. This process leaves a residue known as *vinasse* which consists of water and nonvolatile components. This wastewater or the spent wash is highly concentrated and contains total dissolved solids (TDS) of 50000 mg/l and organic matter. The composition of vinasse has been described by many researchers (Garcia et al., 1997; Gonzalez et al., 2000; Jimenez et al., 2005). It is dark in color, with a typical odor and a strongly acidic nature (pH $\cong$ 3.5), and its organic COD content is high between 10 and 80 g/l. The highest levels of BOD, COD, COD/BOD ratio, phosphorus, and sulfate are found in sugarcane molasses stillage; low levels of COD and BOD are noted in sugarcane juice stillage (Wilkie et al., 2000). The common guppy *Lebistes reticulatus* has been used in the bioassay of distillery effluent (Kumar et al., 1995).

3.3   ALTERNATIVE INDUSTRIAL STILLAGE TREATMENT REACTORS

An understanding of processes that utilize the by-product wastewaters associated with ethanol production is important for treatment. During the last decade, a considerable amount of research has been conducted on the anaerobic treatment of distillery wastewaters. Nine years of experience in designing and operating full-scale anaerobic–aerobic treatment plants with fermentation from sugar beet molasses, together with operating problems and their solutions, has been described (Ciftci and Ozturk, 1995). A two-stage treatment has been used for high-strength wastewaters since 1986: an anaerobic first stage and an aerobic second stage. The anaerobic reactors used are upflow-anaerobic sludge blanket (UASB) reactors with internal and external sludge recirculation facilities.

The major process configurations for high-rate digesters over the past 20 years have been described (Hickey et al., 1991); these include UASB, upflow and downflow stationary packed-bed and fluidized- and expanded-bed reactors. An anaerobic upflow blanket filter has been used in biomethanation (Hanqing and Guowei, 1996) and an unheated anaerobic filter has been used in mesophilic digestion (Leal et al., 1998) of brewery wastewaters. Recently, a novel inverse turbulent-bed bioreactor for anaerobic treatment has been discussed (Buffiere et al., 2000). Akunna and Clark (2000) described the

performance of a granular-bed anaerobic baffled reactor (GRABBR) treating whisky distillery wastewater. Anaerobic fluid-bed technology has been found more effective than anaerobic filter technology for treating distillery wastewater (Perez et al., 1998). Harada et al. (1996) examined the feasibility of thermophilic (55°C) anaerobic treatment of distillery wastewater (sugarcane molasses vinasse) using a 140-L UASB reactor for a period of 430 days. COD and BOD removal during the entire experimental period was 39 to 67% and more than 80%, respectively. Wilkie et al. (2000) described the treatment parameters and efficiencies of mesophilic and thermophilic anaerobic digestion of stillage from beet and cane molasses and other raw materials and listed some of the full-scale anaerobic digesters currently used to treat stillage. Of 149 facilities constructed, 87 are in India. These include 78 UASBs, 3 expanded granular sludge-bed (EGSB) reactors, commissioning of 27 bulk volume fermentors (BVFs), 22 downflow fixed-film (DFF) digesters, 10 anaerobic contact (AC) digesters, 6 hybrid digesters, and 3 upflow fixed-film (UFF) digesters. Singh and Nigam (1995) summarized the effectiveness and limitations of various alternative treatment technologies of distillery effluents in India. Brewery wastewater can be treated anaerobically by the addition of activated sludge in reactors. The potential of EGSB reactors has been explored for anaerobic treatment of low-strength brewery wastewater (Kato et al., 1999).

Physical or mechanical separation was one of the earliest methods used to recover or remove supended solids containing yeast and other materials. After mechanical separation, a number of technologies exist for further processing, such as evaporation, single-cell protein production, and anaerobic digestion. Kim et al. (1999) employed membrane separation for the concentration of stillage. However, a membrane anaerobic reactor treating brewery wastewater results in inert COD production (Ince et al., 2000). In recent years, thermal and electrochemical methods have been examined that show a potential for stillage processing. Catalytic wet air oxidation is used to treat high-strength distillery liquors (Belkacemi et al., 2000). Goto et al. (1999) showed a rapid reduction in organic strength by supercritical water oxidation of distillery wastewater, using H_2O_2 at higher temperatures of 673 to 773 K. Electrochemical treatment of distillery spent wash and vinasse from beet molasses produces chlorine, chlorates, and other oxidants that can destructively oxidize COD (Vlyssides et al., 1997; Vijayaraghavan et al., 1999). Beltran et al. (1999a,b) discussed the degradation of distillery wastewater by oxidative treatment using ozone and an integrated ozone–biological treatment. Vinasse-containing wastewater can be purified by an anion-exchange process using the biopolymer chitosan (Lalov et al., 2000). These methods are not being used at full scale at present and cannot be considered as proven or economical. Scheper et al. (1999) described the principles of bioanalytical systems for online bioprocess monitoring, including the use of bioanalytical tools for process optimization.

3.4   FUNGAL TREATMENT OF DISTILLERY AND BREWERY WASTES

Decolorization of MSW by physical or chemical methods is unsuitable. Also, the mesophilic and thermophilic anaerobic digestions are not free of disadvantages. Despite rapid growth and assimilation of a wider range of substrates by bacteria than fungi, bacterial separation is difficult and expensive, due to their small size, and their nutritive value has not been fully realized. Bacterial use is limited, due to problems in separation, low ethanol tolerance, lower cell yield, and lower feed by-product return, despite higher ethanol yields. Such factors have limited the market for bacterial feed additives. Algae require high intensities of light and large areas.

Degradation of these wastes requires the selection of microorganisms that can assimilate them. Research on the fungal treatment of these wastewaters dates back to the 1960s. Yeasts and fungi have been used for reducing the strength of distillery and brewery wastewaters and molasses with concomitant production of single-cell protein (SCP). Thus, yeasts and fungi are considered the most suitable organisms for these purposes.

Various strains of the yeast *Saccharomyces cerevisiae* are most widely used because of their good growth rate and high ethanol tolerance (Ingledew, 1995). *S. cerevisiae* has been shown to tolerate ethanol concentrations of up to 23% when grown under proper nutrient conditions. Some researchers have advocated the use of thermotolerant yeasts to obtain higher fermentation rates and ethanol yields and reduced requirements for cooling. However, Lynd et al. (1991) reported a low ethanol tolerance of thermophilic fermenting organisms, due to leaky cell membranes at higher temperatures. Higher temperatures can cause higher saturated esterified fatty acids, such as palmitic and pamitoleic acids, in the yeast cell membrane at the expense of unsaturated fatty acids such as oleic, linoleic, and linolenic acids (van Uden, 1984; Suutari et al., 1990). Decrease in the amount of membrane phospholipids to maintain membrane fluidity for cellular activities in *S. cerevisiae* is an adaptive response (Lloyd et al., 1993; Rose, 1993). Certain aspects of stillage utilization, bioconversion, and treatment by fungi have been discussed (Friedrich et al., 1992; Wilkie et al., 2000).

3.5   FUNGAL FERMENTATION AND DECOLORIZATION

Operation of the fermentation process has been classified into three categories: batch, continuous, and partially continuous. In a batch process, cooled mash is inoculated with yeast equivalent to 10% of the fermentor volume and allowed to ferment about, or less than, 48 hours (ASTM, 1997). Completion of the fermentation results in the formation of stillage. Continuous fermentation has many advantages over batch culture. Higher ethanol production with a decreased yield of yeast can be obtained in continuous fermentation using

recycled yeast (Warren et al., 1994) or immobilized yeast (Arasaratnam and Balasubramaniam, 1998). Continuous fermentation lowers the stillage COD when yeast is not recovered. In such instances, yeast production is lower than that of batch fermentation. Continuous fermentation is prone to contamination by microorganisms, which secrete their products and increase the stillage COD. In a partially continuous fermentation, yeast is partially recovered before distillation and returned to fermentation. A decrease in volume of stillage and increase in COD are produced by the volume of yeast returned. Stillage recycling is known to lower stillage volume without affecting total COD. Several researchers have reported a practical limit of 50% stillage recycling (de Menezes, 1989; Shojaosadati et al., 1996; ASTM, 1997). Above this limit, yeast inhibition results in lowering ethanol yield and increasing COD concentration. Fungal fermentation and decolorization of distillery and brewery wastes are noted in Tables 3.1 and 3.2, respectively.

3.5.1   Yeasts

Yeasts have been cultivated in distillery stillages for a long time. Yeasts can grow rapidly and avoid possible contamination in fermentations by other microorganisms. The use of yeast is important, due to high protein and vitamin contents. Studies in 1960s involved the use of many species of *Candida* in the conversion of sugarcane and beet molasses distillery effluents. Of 203 yeast strains, Akaki et al. (1981) selected *Hansenula*, *Debaryomyces*, and *Rhodotorula* for the treatment of sugarcane molasses stillage. An enrichment program resulted in fermentation of sugarcane molasses at 43°C in batch experiments, with the sugar conversion efficiency exceeding 94% (Abdel-Fattah et al., 2000). The two best performers, *Saccharomyces cerevisiae* F111 and *Kluyveromyces marxianus* WR12, produced mean ethanol of 7.7% and 7.4% (w/v), respectively, coupled with the advantage of cooling elimination during fermentation. Batch fermentation showed a diauxic form of cell growth after 18 hours that could be attributed to a change in the enzyme system related to degradation and absorption of macromolecular components of the sugar beet stillage (Shojaosadati et al., 1999). In a continuous culture, COD was reduced 31% without the addition of nutrients in sugar beet stillage. COD reduction and substrate utilization in three different beet stillages occurred during growth by a thermotolerant yeast, *Candida rugosa* (Lee, 1991). Of three yeast isolates, MEA5 removed 95% and 46% of the COD of synthetic winery wastewater within 24 hours under aerated and nonaerated conditions, respectively (Malandra et al., 2003). Three yeast isolates reduced the COD 60% after 120 hours. Mixed biofilms of yeasts and bacteria in rotating biological contactor (RBC) reduced the COD 43% in 1 hour.

Of 205 yeast strains, *Citeromyces* sp. WR-43-6 decolorized 68.91% of the molasses pigment solution at 30°C and pH 6.0 containing 2% glucose, 0.1% sodium nitrite, and KH_2PO_4 (Sirianuntapiboon et al., 2004). This strain also decolorized 75% of the alcohol factory stillage and reduced the COD 100%

TABLE 3.1 Fungal Fermentation of Distillery and Brewery Wastes

Fungus	Waste Type/ Dilution	Mode of Cultivation	COD Reduction [% (max.)]	TOC Reduction [% (max.)]	Biomass	Protein Content (%)	Duration (days)	Reference
Yeasts								
Hansenula sp.	Sugar beet stillage	Continuous	31		$5.7\,g/dm^3$	39.6	2	Shojaosadati et al., 1999
Candida rugosa	Sugarcane stillage	Batch	41		20.4g/l	30.4	2	Lee and Lee, 1995
I-44	Sugar beet stillage 1:5	Batch		49			3	Moriya et al., 1990
Hansenula anomala J 45	1:10			39				
H. anomala J 45-N-5	1:7.5	Batch		51			1	Malandra et al., 2003
H. fabianii J 640	1:10			31			2	
H. fabianii J 640-4-1	1:7.5			45			2	
MEA5	Winery synthetic wastewater	Batch		95			1	
MEA4				46			2	
MEA9				69			2	
Citeromyces sp. WR-43-6	Alcohol stillage	Batch		100			8	Sirianuntapiboon et al., 2004
Filamentous fungi								
Aspergillus terreus	Vinasse	Batch	29	28			5	Garcia et al., 1997
Geotrichum candidum			28	26				
Aspergillus niger	Sugarcane vinasse	Batch	74	40				Vitali and Kiyan, 1996
A. niger	Citrus vinasse		60					
Aspergillus sulphureus			60					
Aspergillus awamori var. *kawachi*	*Shochu* stillage	Jar-fermentor		44	40g/l	40	1–2	Morimura et al., 1994b
White-rot fungi								
Flavodon flavus	Cane molasses spent wash/10%	Batch	80				8	Raghukumar and Rivonkar, 2001
Trametes sp. I-62	Molasses vinasse/20%	Batch	62				7	Gonzalez et al., 2000

TABLE 3.2 Fungal Decolorization of Distillery and Brewery Wastes

Fungus	Stillage Origin/ Dilution	Mode of Cultivation	Decolorization (%)	Enzyme Production (Activity)	Duration (days)	Reference
Geotrichum candidum Dec 1	Molasses/diluted	Batch	87	Peroxidase (low)	12	Kim and Shoda, 1999
Coriolus versicolor	Molasses spent wash/ 12.5%	Batch	53		10	FitzGibbon et al., 1998
Flavodon flavus	Cane molasses spent wash/10% and 50%	Batch	80	MnP, laccase	8	Raghukumar and Rivonkar, 2001
Phanerochaete chrysosporium JAG-40	Synthetic melanoidin solution	Batch	80	Extracellular enzymes	6	Dahiya et al., 2001
P. chrysosporium ATCC 24725	Molasses spent wash/ 6.25%	Batch	85		10	Fahy et al., 1997
Trametes sp. I-62	Distillery vinasse/20%	Batch	73	Laccase (35 times)	7	Gonzalez et al., 2000
Coriolus hirsutus (pellets)	Melanoidin-containing medium	Batch	60	MnP and MIP, H_2O_2, laccase	5	Miyata et al., 1998
Citeromyces sp. WR-43-6	Alcohol stillage	Batch	60–70		8	Sirianuntapiboon et al., 2004
Penicillium decumbens	Vinasse	Batch	41		4	Jimenez et al., 2005

and the BOD 76%. During a periodic feeding system, *Citeromyces* sp. WR-43-6 reduced the color intensity 60 to 70%, the COD 99% and the BOD 91% over an 8-day cultivation period on 10% fresh medium. The strain showed a constant decolorization of 75% during a four-step replacement culture system.

3.5.2   Filamentous Fungi

Filamentous fungi grow slowly and are susceptible to contamination. However, filamentous fungi exhibit certain advantages, as they produce several extracellular hydrolytic enzymes for the assimilation of complex carbohydrates without prior hydrolysis. This quality makes possible the utilization of complex substrates. Another advantage is the easy separation of biomass by filtration due to the filamentous structure. In comparison to yeasts, filamentous fungi are less prone to variations in nutrients, aeration, temperature, and pH and have a lower nucleic content in the biomass.

Vinasse contains organic matter that includes more than 10 phenol compounds and their polymers. These compounds are difficult to biodegrade and hamper removal by anaerobic digestion. It appears that prior removal of these compounds can expedite anaerobic digestion, thereby reducing cost through a shortened water retention time. Anaerobic treatment can be improved by prior removal of phenolic compounds due to aerobic biological treatment (Maestro-Duran et al., 1993). The pretreatment reduced COD, lowered toxicity, and diminished phenol content, which makes it amenable to anaerobic treatment.

Aspergillus terreus removed about 66% of total phenols and 94% of *ortho*-diphenols after 100 to 120 hours of fermentation (Garcia et al., 1997). Preliminary experiments implied that the end of fermentation was caused by depletion of nitrogen in the medium. *Geotrichum candidum* removed about 70% of total phenols and 91% of *ortho*-diphenols. These results suggest the aerobic degradation of phenol compounds in vinasse without the addition of nutrients in the medium. Biomass content depends on the removal of phenols. The peroxidase activity of *G. candidum* Dec 1 was lower, due to the inhibitory effect of molasses, but the inhibition decreased after the attainment of full fungus growth (Kim and Shoda, 1999).

Of four fungi, *Coriolus versicolor* revealed the highest color removal, with a reduction of 0.43 unit at A_{475} (equivalent to 53% color reduction) after 10 days in the presence of 12.5% (v/v) MSW (FitzGibbon et al., 1998). Giant colony and shaking culture methods were employed for the primary and secondary screening of the fungi to decolorize the melanoidins (FitzGibbon et al., 1995). The primary screening indicated a color change from black to light brown in 10 days by *C. versicolor* and *Mycelia sterilia*. Secondary screening showed the ability of all fungi to reduce COD (55 to 70%) of spent wash.

A bioconversion process for the treatment of fruit distillery wastes using filamentous fungi has been discussed (Perdih et al., 1991). *Phanerochaete*

chrysosporium and *Trichoderma reesei* produced filter cakes of 22 and 17% raw protein contents by degrading raw fibers by 20% (Friedrich et al., 1986). Species of *Aspergillus* revealed better filtration time and COD reduction and showed the same efficiency in protein synthesis to *P. chrysosporium* and *T. reesei*, but did not degrade fibers. Due to slow growth, *Pleurotus ostreatus* did not compete with other fungi. Of 228 strains, nine revealed decolorization exceeding 50% and required different culture conditions (Sirianuntapiboon et al., 1988a). The strain D90 showed the highest decolorization yield, 93%, in 8 days in molasses wastewater containing 2.5% glucose, 0.2% yeast extract, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$. *M. sterilia* D90 showed the highest decolorization, 90%, and a BOD of 80% in 10 days in the presence of nutrients such as 2.5% glucose, 0.2% $NaNO_3$, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ (Sirianuntapiboon et al., 1988b). The strain decolorized 70% in 11 days and eliminated 90% of BOD in 15 days under nonsterile conditions. About 75% molasses melanoidin solution decolorized on glycerol-peptone medium at 45°C in 3 days with shaking using a thermophilic strain, *Aspergillus fumigatus* G-2-6 (Ohmomo et al., 1987). Continuous decolorization experiments in a jar-fermentor resulted in a constant decolorization of 70% at a dilution rate of 0.014 per hour coupled with elimination of about 50% COD and 56% TOC. Five filamentous fungi reduced up to 60% COD of rum stillage; *Gliocladium deliquescens* performed best (Barker et al., 1982). Of various filamentous fungi, *Aspergillus niger* and *A. oryzae* exhibited high COD reduction and the highest biomass (de Lamo and de Menezes, 1978).

3.5.3 White-Rot Fungi

White-rot fungi are the most significant lignin degraders in nature. In the 1990s, several researchers used white-rot fungi in the decolorization of distillery effluents. Decolorization appeared to be attributed to the secretion of extracellular ligninolytic enzymes. A marine basidiomycete and the white-rot fungus *Flavodon flavus* showed good salt tolerance and reduceed color from the raw MSW about 70 to 80% at a 10% concentration on LN, ME broth, and sugarcane bagasse media on day 8 (Raghukumar and Rivonkar, 2001). Decolorization activity was also detected in media prepared with half-strength seawater of up to 15 ppt salinity. A two- or threefold reduction in mycelial dry weight and the production of lignin-degrading enzymes such as manganese peroxidase (MnP) and laccase was detected in the presence of raw MSW. A negative correlation exists between MnP production and MSW decolorization. Extracellular enzymes were responsible for the decolorization of synthetic and natural melanoidins in spent wash using *Phanerochaete chrysosporium* JAG-40 (Dahiya et al., 2001). Gel-filtration chromatography has depicted more rapid decolorization of large-molecular-weight fractions of melanoidins than of small-molecular-weight fractions. Watanabe et al. (1982) reported 80% melanoidin decolorization by a strain of *Coriolus*. About 49% of the color was eliminated in the absence of either glucose or peptone by *P.*

chrysosporium (Fahy et al., 1997). This seems to be the first report that shows decolorization of MSW in the absence of an additional carbon source. Under optimum conditions, *Coriolus versicolor* Ps4a shows the highest decolorization yield, 80% (Aoshima et al., 1985). A good correlation exists between decolorization activity and mycelial growth. The decolorization activity is due to decomposition of the molasses pigment and intracellular enzymes.

Molasses pigments are also decolorized by ligninolytic cultures of *P. chrysosporium* (Durrant, 1996). Production of lignin peroxidase (LiP) increases with the addition of molasses to the culture medium. Simultaneous color removal occurs with the appearance of LiP, and the rate of color removal is directly proportional to the activity of LiP. Repeated production of LiP and decolorization of pigment result by immobilizing the fungus in polyurethane foam, providing the possibility of improving the process. *Phanerochaete chrysosporium* also produceed LiP in a culture medium containing molasses (Jafelice et al., 1990).

Trametes sp. I-62 (CECT 20197) treatment removed 73% of the color and 62% of the COD after 7 days in the presence of 20% (v/v) of distillery vinasses in the medium (Gonzalez et al., 2000). Such conditions resulted in a 35-fold increase in laccase production by *Trametes* sp. I-62. A number of pyrolysis products, such as furan derivatives, were reduced after 7 days of fungal treatment. Vinasse color removal appeared to be associated with melanoidin degradation.

3.5.4 Mixed Cultures

Several fungi have been used in pure and mixed cultures for the conversion of stillage. This can improve the assimilation of nutrients in stillage and provide better yields of biomass. Combinations of different yeasts, molds, and filamentous fungi; yeasts and molds; yeasts and filamentous fungi; and yeasts and bacteria are used. Mixed cultures related to the bioconversion of various stillages have been noted (Friedrich et al., 1992). Several yeasts, molds, and bacteria have been employed for the aerobic treatment of sugarcane stillage (Nudel et al., 1987). Using cultures of two yeasts and a mold, *Candida utilis* showed a rapid increase in biomass during early stages of growth and established itself until the end of the process. Mixed cultures of *C. utilis* with *Corynebacterium acetoacidophilum* and of *C. utilis* with *Brevibacterium flavum* are known to increase the amino acid and vitamin contents of the biomass. *C. utilis* with *Azotobacter* is a good combination. *Azotobacter* can prevent nitrogen addition in stillage, and the increased biomass is related to nitrogen fixation by bacteria.

A two-step continuous fermentation has also been used for the conversion of stillage. *C. utilis* produced high quantities of biomass during the first step, and *Aspergillus niger* reduced the COD of the effluent during the second step (Nudel et al., 1987). Of five filamentous fungi, *Trichoderma viride* and *Paecilomyces variotii* proved to be the most effective organisms. A two-step

continuous aerobic process was suggested for the treatment of cane stillage (Castlla et al., 1984). During the first step, the original stillage was employed for the cultivation of *C. utilis*, and the decanted supernatant was used during the second step, where a strain of *Paecilomyces* was cultivated without the addition of nutrients. A total COD reduction of 87% and a mixed biomass output of 2.3 to 2.6 g/l per hour, mostly *Candida*, were achieved. A two-stage process for beet molasses, with *Hansenula anomala* J 45-N-5 followed by an unknown soil yeast isolate I-44, resulted in a 75% reduction in COD (Cabib et al., 1983).

Malnou et al. (1987) showed a 54.9% reduction in the COD of whiskey stillage using a mixed culture of *Geotrichum candidum*, *Candida krusei*, and *H. anomala*. A mixed culture of a filamentous fungus and two yeasts was employed for protein production in whiskey distillery spent wash (Barker et al., 1982). Contamination was also prevented by the use of mixed cultures in continuous fermentation. *Candida krusei* was the dominant organism during the early stage and at high dilution rates and used protein as both carbon and nitrogen sources. *G. candidum* and *H. anomala* assimilated carbohydrates, glycerol, and lactate.

3.6   MOLASSES TOXICITY TO FUNGI

Increased tolerance of toxic substances from beet molasses results in changes in the development and ultrastructure of *Aspergillus niger* mycelium, strain Z (Zakowska and Gabara, 1991). Toxic molasses compounds and Spumol BJ at a concentration of 5 μl per 100 cm^3 produced conidia swelling. Giant conidia were unable to germinate and the mycelium began sinking after 24 hours. Electron microscopic examination revealed the electron-transparent cytoplasm with a decrease in ribosome number and changes in the ultrastructure of mitochondria. This may be attributed to a disturbance of respiratory processes in the mycelium. The appearance of electron-dense deposits in mitochondria implies the onset of a defense mechanism, eliminating toxic substances. Cell walls of mycelium of *A. niger* strains sensitive or resistant to toxic molasses growing in the presence of Spumol K have also been examined (Gabara and Zakowska, 1997).

3.7   FACTORS AFFECTING FUNGAL FERMENTATION AND DECOLORIZATION

Several factors, such as sources of carbon, nitrogen, and phosphorus; pH; temperature; dilution of the effluent; inoculum dose; and static versus agitated culture conditions, are known to influence the rate of decolorization and COD removal of distillery and brewery effluents by fungi (Table 3.3). These factors are described below.

TABLE 3.3 Influence of Supplements on Fungal Fermentation and Decolorization of Distillery and Brewery Wastes

Fungus	Stillage Origin/ Dilution	Supplements	COD Reduction (%)	Decolorization (%)	Duration (days)	Reference
Flavodon flavus	Cane, MSW/10%	Glucose or sucrose		63–64	6	Raghukumar and Rivonkar, 2001
Phanerochaete chrysosporium ATCC 24725	MSW/6.25%	Glucose		85	10	Fahy et al., 1997
Coriolus versicolor	Digested	Glucose	90	71.5	12	Kumar et al., 1998
P. chrysosporium	Molasses spent wash/6.25%	Yeast extract	73	53.5		
Trametes versicolor	Beet molasses/ undiluted	Sucrose MnSO$_4$	77 70	84 76	4	Benito et al., 1997
Aspergillus niger	Beet molasses/ undiluted	Sucrose MnSO$_4$ KH$_2$PO$_4$ NH$_4$NO$_3$	75	69	4	Miranda et al., 1996
Hansenula sp.	Beet stillage/ undiluted	NH$_4$SO$_4$ KH$_2$PO$_4$	36		2	Shojaosadati et al., 1999

3.7.1 Carbon Source

The availability of a carbon source is a requirement for the growth of fungi and melanoidin removal. The spent wash contains little readily available carbon despite high total sugar content. Of various carbon sources, glucose provides the highest growth and decolorization rates. Of several carbon sources (e.g., pentoses, hexoses, and sugar alcohols), glucose or sucrose proves best for decolorization of raw MSW at 10% concentration in LN medium using *Flavodon flavus* (Raghukumar and Rivonkar, 2001). Optimum growth and decolorization of digested MSW occur at up to 3% glucose (w/v) for *Phanerochaete chrysosporium* and up to 5% (w/v) for *Coriolus versicolor* (Kumar et al., 1998). The decolorization activity correlates well with the growth of mycelia of *A. niger* (Miranda et al., 1996).

3.7.2 Nitrogen and Phosphorus Sources

Nitrogen is a component of cell wall polymers and is important for protein synthesis. A low-nitrogen medium favors the highest decolorization of MSW by *F. flavus* (Raghukumar and Rivonkar, 2001). Shojaosadati et al. (1999) reported a COD reduction of 35.7% by the supplementation of $4\,g/dm^3$ ammonium sulfate and $0.4\,g/dm^3$ KH_2PO_4 to sugar beet stillage in a continuous culture of *Hansenula* sp. The highest productivity is achieved in a dilution rate of 0.12 per hour. No doubt, supplementation of these nutrients increases the levels in COD reduction and biomass production, but its use depends on the overall economic evaluation of the process. Among the various nitrogen sources, yeast extract and peptone were shown to support the highest growth and decolorization by *Coriolus versicolor* and *Phanerochaete chrysosporium*, respectively (Kumar et al., 1998). Of various additives, phosphoric acid was shown to provide the best protein and biomass production in *Candida krusei* (Ruegger and Tauk-Tornisielo, 1996). *Candida rugosa* reached a maximum of $T_{X/COD}$ (biomass production/COD reduction) and $Y_{CPP/COD}$ (crude protein production/COD reduction) at a concentration of $2\,g/l$ of urea in a shaking culture at 4°C for 2 days (Lee and Lee, 1995). At this concentration, the yeast showed the highest biosynthetic rate and the lowest energy maintenance. The highest decolorizing activity of strain D90 occurs in the presence of yeast extract, peptone, and sodium nitrate as the nitrogen sources (Sirianuntapiboon et al., 1988a).

The highest color removal occurred in the absence of the addition of ammonium nitrate by *Trametes versicolor* (Benito et al., 1997). The COD removal rate was 65 to 70%. The color elimination and COD reduction were similar and more or less unaffected by the various manganese and magnesium concentrations. About 80% of the COD was removed in the presence of $1\,g/l$ of KH_2PO_4 compared to 53% in the absence of phosphate in the wastewater. An addition of $1\,g/l$ of potassium phosphate caused 68% color elimination and 72% COD removal using *Aspergillus niger* (Miranda et al., 1996). About

68% of the color was eliminated by the addition of $0.5\,g/l$ $MgSO_4 \cdot 7H_2O$ to the wastewater. The COD removal was 65 to 70%, which remained almost unaffected by magnesium concentration. Color removal was enhanced from 34% to 68.5% by the addition of $1.8\,g/l$ of ammonium nitrate.

3.7.3   Temperature

Temperature influences growth, metabolism, nutrition, enzymes, biomass, and cell permeability. Temperature control generally adds a cost to an industrial operation. Thus, it is important to determine the temperature range where fungi can show the maximum growth rate or product formation. Based on the continuous culture of *Hansenula* sp. at various temperatures, the temperature range 32 to 36°C was shown to be optimum for activities (Shojaosadati et al., 1999). This temperature also provided better flocculation characteristics, which would improve the recovery cost. However, a sharp decline in growth was found beyond these deviating temperatures. The optimum growth and highest color removal occurred at 30°C for the strain D90 (Sirianuntapiboon et al., 1988a), 35°C for *Coriolus versicolor*, and 40°C for *Phanerochaete chrysosporium* (Kumar et al., 1998). These temperatures are also optimum for the enzymes responsible for decolorization. *Candida rugosa* grows faster with a COD reduction rate of 45.5% and a biomass yield of $14.7\,g/l$ at 40°C (Lee and Baerwald, 1991).

3.7.4   pH

The pH plays an important role in the decolorization process because some of the components of wastewater color are soluble over a certain range of basic pH and insoluble at acidic pH. *C. versicolor* and *P. chrysosporium* promote the highest color removal, 52% and 33.5% at pH 5.0, respectively (Kumar et al., 1998). The best decolorization (80%) and highest COD removal (75%) were shown to occur at an initial pH of 5 (Benito et al., 1997). The initial pH does not affect the percentage removal of NH_4^+—N. The reduction in the ammonium concentration ranged from 25 to 35%. Total color elimination was 69% at an initial pH of 5.0 (Miranda et al., 1996). All isolates, including strain D90, exhibited a high level of decolorizing activity at pH 5 to 6 (Sirianuntapiboon et al., 1988a).

3.7.5   Agitation and Aeration

The availability of oxygen is one of the most important factors for the industrial production of yeasts. Agitation and aeration are two factors that can affect the oxygen transfer rate in cultures. Agitation affects oxygen and substrate uptake, consumption of energy, production of biomass, and overall production costs. Agitation affects gas–liquid mass transfer in three ways: (1) increasing the contact area by dispersion of small bubbles, (2) increasing the

gas–liquid contact time through the whirling movement, and (3) reducing the consistency of liquid film through turbulence.

Biomass production and COD reduction by *Hansenula* sp. were shown to increase with an increase in agitation speed, and the maximum performance reached at 900 rpm (Shojaosadati et al., 1999). A higher agitation speed can cause disruption of fungal cell walls, thereby decreasing performance. Aerobic batch fermentation is conducted at a constant speed of 500 rpm for *Aspergillus terreus* and *Geotrichum candidum* (Garcia et al., 1997). Another important factor is aeration that affects the oxygen transfer rate by increasing the partial oxygen pressure. The optimum aeration rate for the production of maximum *Hansenula* sp. biomass and COD reduction was shown to be 1.5 (v/v/m) (Shojaosadati et al., 1999). The best decolorization of MSW has been demonstrated by *Flavodon flavus* in oxygenated cultures (Raghukumar and Rivonkar, 2001). A shaking rate of more than 100 oscillations/min was sufficient for a decolorization yield of 60 to 65%, which is equivalent to 1 ppm of dissolved oxygen (Ohmomo et al., 1985).

3.7.6 Inoculum Size

Few researchers have focused on the effect of inoculum size on the decolorization and COD reduction of MSW. Dahiya et al. (2001) demonstrated a 5% (w/v) dry weight of mycelial supension of *Phanerochaete chrysosporium* JAG-40 to be optimal for maximum decolorization in melanoidin medium supplemented with glucose and peptone. Color elimination and COD reduction occurred with different inoculum mycelial concentrations in batch cultures of *Trametes versicolor* (Benito et al., 1997). Similar yields of color elimination (80%) were obtained at mycelia concentrations of 1.5 to 2 g/l. Higher mycelia concentrations started biodegrading after 2 days of culture, with lower mycelia concentrations the third day. The COD removal ranged from 72 to 75% and remained unaffected by the initial mycelia concentrations. A constant decolorization yield of 75% occurred after 6 days of culture of strain D90 at the rate of inoculum size of 0.15 g of mycelia per 100 ml of medium (Sirianuntapiboon et al., 1988a). The optimum mycelia mass of *Coriolus versicolor* Ps4a for decolorization was 1.2 g dry weight (Ohmomo et al., 1985).

3.7.7 Effluent Dilution Rate

The dilution rate affects biomass yield and productivity. Productivity is a function of cell concentration in a fermentor and the specific growth rate. The effect of effluent dilution rate on decolorization and COD removal by fungi was summarized in the foregoing paragraphs. The treatment of distillery wastewater at various dilutions by yeasts has been described by Moriya et al. (1990).

3.8   MECHANISMS OF MELANOIDIN DEGRADATION

Molasses is difficult to decompose by the usual biological treatment processes, due to the presence of melanoidins. Melanoidin is a complex polymer formed by Maillard aminocarbonyl reaction products, and its chemical structure remains unknown. Despite wide distribution in food and preservation processes, melanoidins are not easily degraded by microorganisms, including fungi. The role of enzymes other than laccase or peroxidases in melanoidin decolorization by strains of *Coriolus* (*Trametes*) was documented during the 1980s. Aoshima et al. (1985) claimed the induction of intracellular enzymes by *Coriolus versicolor* Ps4a in the presence of melanoidin. Two-thirds of enzymes may be sugar dependent and the other one-third independent of sugar. The sugar-dependent enzymes seem to be the same as sorbose oxidase from *Coriolus* sp. no. 20 or a similar kind of sugar oxidase (Watanabe et al., 1982). The decolorization of melanoidins may be caused by active oxygen produced by reaction with these sugar oxidases.

Little is known about the role of fungal ligninolytic enzymes in decolorization of distillery effluents. A culture filtrate of *T. versicolor* showed a mineralizing activity on melanoidins (Dehorter and Blondeau, 1993). A 47-kDa extracellular protein carried out the mineralization of melanoidins, and this Mn(II)-dependent system required oxygen and was inhibited by the inhibitors of heme proteins. However, oxidation did not occur through the formation of hydrogen peroxide in the presence of melanoidins as substrate. Cell-free extracts from *T. versicolor* also showed Mn(II)-dependent enzyme activity on melanoidins. *Trametes* sp. I-62 produced laccase activity as a result of a response to the oxidizing of compounds in distillery vinasse, and this diminished their potential toxic effects (Mansur et al., 1997). Gonzalez et al. (2000) reported a laccase activity of 0.02 U/ml by *Trametes* sp. I-62 in Kirk's medium and 0.7 U/ml in Kirk's medium supplemented with 20% vinasses. This 35-fold increase in laccase activity may imply an enhancement of laccase production by some components of vinasses. However, laccase activity was enhanced twofold by *Trametes* sp. I-62 containing 20% effluent (Mansur et al., 1997). A higher increase in laccase activity may be related to decolorization of the effluent (Gonzalez et al., 2000). Miyata et al. (1998) reported enzymatic decolorization of melanoidins by *Coriolus hirsutus* pellets. This is one of the more complete enzymatic studies on the decolorization of melanoidins. Synthetic melanoidins are decolorized by the participation of manganese peroxidase (MnP) and manganese-independent peroxidase (MIP) and the extracellular H_2O_2 produced by glucose oxidase along with the partial participation of laccase.

Decolorization of sugar refinery effluent by *Phanerochaete chrysosporium* coincided with the time of detection of MnP in the medium (Guimaraes et al., 1996). *P. chrysosporium* JAG-40 produces extracellular enzymes, which resulted in a 40 to 50% color removal of synthetic melanoidins in 24

hours (Dahiya et al., 2001). The culture filtrate was used as the enzyme source for melanoidin decolorization. Kumar et al. (1998) reported on decolorization as a result of secondary metabolic reaction from a secondary metabolite by white-rot fungi. But the nature of the secondary metabolite is unknown. Increased glucose oxidase activity correlates with the increasing decolorization of MSW by free mycelia of *Flavodon flavus* (Raghukumar et al., 2004). It has been proposed that glucose oxidase activity leads to the formation of hydrogen peroxide, which acts as a bleaching agent on the MSW. Further studies on the contribution of MnP, LiP, MIP, or intracellular H_2O_2-producing enzymatic systems for the degradation of melanoidins are required. In addition, the role of other enzymes and some mediators or inducers for color removal need to be investigated.

Color adsorption is also one of the mechanisms of melanoidin decolorization. The color adsorption onto mycelia of *T. versicolor* (Benito et al., 1997) and *Aspergillus niger* (Miranda et al., 1996) occurs throughout the experiments. The percentages of color adsorbed on *T. versicolor* and *Aspergillus niger* mycelia are 5 to 10 and 10 to 25%, respectively. About 83% decolorization and 17% adsorption on the mycelium of *A. niger* occur in optimum nutrient culture concentrations. Benito et al. (1997) revealed 90% decolorization and 10% adsorption on the mycelium of *Trametes versicolor* in optimum nutrient culture concentrations. High decolorization activity occurred by adsorption of melanoidins to the mycelium of a strain of *Aspergillus fumigatus* G-2-6 (Ohmomo et al., 1987).

3.9   FUNGAL BIOREACTORS FOR DISTILLERY AND BREWERY WASTES

Fungal conversion of distillery stillage usually employs submerged processes, which are conducted in shaken flasks and fermentors. Stirred-tank bioreactors have been in use for the last two decades. Column fermentors have also been employed by several researchers. The volume of laboratory fermentors ranges from several hundred milliliters to several liters. Batch and continuous modes of operation are well established. A two-stage continuous cultivation with one fungus or two different fungi or microorganisms is also recognized. In certain cases, sterile conditions are necessary, and in others, such conditions are not required. Antifoam agents or mechanical devices are used to disrupt foam during fermentation. Different rates of aeration are used in different types of bioreactors. The dilution rates are generally tested in continuous cultivation and two-stage continuous processes. After fermentation, the yeast biomass can be separated from the broth by centrifugation and in filamentous fungi by filtration and screening. Table 3.4 lists the important bioreactor types used for the decolorization of distillery and brewery wastes.

TABLE 3.4 Fungal Bioreactors for Decolorization of Distillery and Brewery Wastes

Bioreactor Type	Fungus	Total Capacity/ Working Vol.	Decolorization (%)	COD Removal (%)	Hourly Productivity (g/l)	Cell Yield (g cells/g sugars)	Duration (hours/ days)	Reference
Fed-batch (stirred-tank)	*Saccharomyces cerevisiae*	12 L			2.33	0.46	20h	Win et al., 1996
Fed-batch	*Mycelia sterilia* D-90	1 L	80	34			8d	Sirianuntapiboon et al., 1988b
Bubble column	*Aspergillus niger*	7 L	37	69			11d	Miranda et al., 1996
	Coriolus versicolor Ps4a	26 × 400 mm	75				80h	Ohmomo et al., 1985
Immobilized in Ca–alginate gel			66	53			16d	Fahy et al., 1997
Immobilized in Ca–alginate gel	*Phanerochaete chrysosporium*		59				10d	FitzGibbon et al., 1995
Packed-bed Immobilized in nylon cubes	*Coriolus versicolor*	3.6 × 48.5 cm		77			10d	
Immobilized in polyurethane foam cubes	*Flavodon flavus*		73				7d	Raghukumar et al., 2004

3.9.1   Fed-Batch Bioreactors

Saccharomyces cerevisiae grows on sugarcane molasses in batch and fed-batch cultivation (Win et al., 1996). The temperature, pH, agitator speed, and airflow rate used in the batch reactor were 30°C, 5.5, 500 rpm and 2.5 l/min, respectively. The productivity and overall cell yield on molasses were 0.22 g/l per hour and 0.18 g of cells per gram of sugar, respectively. In a fed-batch stirred-tank reactor, a productivity of 2.33 g/l per hour and an overall cell yield of 0.46 g of cells per gram of sugars were obtained. However, the ethanol concentration was 0.45 g/l and the biomass yield was 44.2 g/l at the end of growth. The fed-batch system was also used for the decolorization of molasses wastewater by *Mycelia sterilia* D90 (Sirianuntapiboon et al., 1988b). The fungus revealed a sufficient and constant decolorization activity of 80%, 79%, and 79% in the first, second, and third batches, respectively. The BOD value was also reduced to 25.8%, 25%, and 34.2% in the first, second, and third batches, respectively. The system reduced the constant value of nitrate in the molasses wastewater.

3.9.2   Bubble Column Bioreactors

The color of molasses wastewater can be reduced continuously in a bubble column reactor using *Aspergillus niger* (Miranda et al., 1996). During this process, the maximum color removal was 37% and was maintained constant for 4 days, and the decolorization activity declined thereafter. The maximum oxygen consumption was related to the maximum decolorization activity and the lowest pH value. However, the decolorization activity decreased and the dissolved oxygen increased after 4 days of continuous operation. In a bubble column bioreactor using mycelia of *Coriolus versicolor* Ps4a, 75% decolorization was shown at a dilution rate of $0.03\,h^{-1}$ in 20 hours under optimum conditions (Ohmomo et al., 1985). Immobilized mycelia in calcium–alginate gel exhibited constant decolorization of 65.7% for 16 days at a dilution rate of $0.22\,h^{-1}$; 53% of the COD and 46% of the TOC were also removed.

Yeasts are also used in aerated reactors to process effluents of high volumetric load (Malnou et al., 1987). Despite the high salinity and COD values of sugar beet molasses, yeasts can be employed to process high volumetric loads exceeding 80 kg of COD per cubic meter per day, reducing 70% of the COD.

3.9.3   Fluidized-Bed Bioreactors

An efficient process has been developed for the utilization of the *Shochu* distillery wastewater using *Aspergillus awamori* var. *kawachi* (Kida et al., 1995). The jar-fermentor studies were carried out aerobically (1 v/v per minute of aeration) at 35°C in a 30-L fermentor (at a working volume of 18 L) with an agitator (250 rpm agitation) containing distillery wastewater as the medium.

The crude protein content of the harvested mycelium was about 40% (w/w) on a dry basis and could be employed as a protein supplement in livestock feed. An anaerobic fluidized-bed bioreactor was used for the treatment of cultural filtrate in the process above was diluted three times with tap water. A 70% TOC removal efficiency was maintained with a loading rate of 22 g/l per day. Methane gas evolution increased with an increase in the TOC loading rate and reached a level of 24.9 L per day. The anaerobically treated wastewater was fed to denitrification and nitrification reactors connected in series that utilize the acclimatized denitrifying and nitrifying bacteria. Removal efficiencies of NH_4^+ (97%) and TOC (94%) could be maintained for 30 days. The effectiveness of the nitrification reactors suggests the suitability of the discharge of the effluent to a river. In addition, this effluent may be used as a liquid fertilizer because of the presence of nitrate and phosphate.

3.9.4   Immobilized Bioreactors

Cells of *Phanerochaete chrysosporium* immobilized in calcium–alginate beads resulted in a much more rapid decolorization of MSW than did free cells (Fahy et al., 1997). Maximum color reduction occurred between 0 and 2 days. However, the color elimination was reduced from 85% with free cells to 59% with immobilized cells after 10 days. The immobilized *Coriolus versicolor* on nylon cubes in a packed-bed bioreactor eliminated the COD of the pretreated spent wash by a further 50.3%, reaching a total reduction of 77% (FitzGibbon et al., 1995). Only 4% color was eliminated, and this was due primarily to absorption onto the fungal mycelia rather than enzymatic oxidation. It is possible to bioremediate such spent waste using a multistage treatment process with an initial treatment with *Geotrichum candidum*. In one study it was shown that immobilized *Flavodon flavus* in 1 cm^2 of polyurethane foam could be used effectively for three consecutive cycles of decolorization of fresh 10% MSW (Raghukumar et al., 2004). The fungus also removed about 98% of the toxicity of the MSW using an estuarine fish, *Oreochromis mossambicus*. Benzo[a]pyrene was present in the MSW and appeared to be one of the causes of toxicity. The total PAH concentration in the MSW was reduced by 68% after 5 days. This is a first report of decolorization of MSW along with simultaneous detoxification and reduction in the PAH content of the MSW.

Four fungi—*Penicillium* sp., *Penicillium decumbens*, *Penicillium lignorum*, and *Aspergillus niger*—produced maximum decolorization of beet molasses alcoholic fermentation wastewater on the fourth day of treatment in a study by Jimenez et al. (2003). *P. decumbens* produced a maximum decolorization of 40%. Four fungi reduced an average of 70% of the phenolic content of the wastewater. *Penicillium* sp. and *P. decumbens* removed 52.1% and 50.7% of the COD, respectively, on the fifth day of fermentation. Anaerobic digestion of previously fermented *P. decumbens* beet molasses was carried out in suspended cell bioreactors. This treatment removed an average of 93%

COD with a methane yield coefficient of 305 ml of methane at STP per gram of COD removal. This combined aerobic–anaerobic treatment removed 96.5% of the COD and reduced the HRT to eliminate COD.

The VFA/alkalinity ratio has been used as a measure of process stability (Balaguer et al., 1992). The VFA/alkalinity ratio was 1.4 and 0.6 for untreated and fermented molasses, respectively, which suggests the removal of volatile fatty acids from the respective wastewater by anaerobic biomasses, with the system reaching equilibrium. Anaerobic digestion of untreated and treated molasses followed the first-order kinetics for biomass loading rates in the range 0 to 0.55 and 0 to 0.75 g of COD per gram of volatile suspended solids (VSS) per day, respectively. Strong brewery waste has been trickled through a vertical curtain of two 3 mm layers of reticulated polyurethane foam bonded to a reinforcing nylon cloth core (Henry and Thomson, 1993). *Geotrichum fragrans* adhered tenaciously to the curtain and acted as a matrix for other yeasts or bacteria. A thick lawn was produced by this process in which a lack of nutrition to cells forced impairment of the curtain. This curtain configuration acts as a self-reproducing cell immobilization on a solid support. Ninety-four percent of the COD was reduced (from 55000 mg/l to 3300 mg/l) by passing through 6 m of curtain. A 1 m^2 curtain with a 4-m fall can treat 15 l/d.

3.10   MODELING

A mathematical model has been developed to compare the behavior of fungi and prediction of the removal of phenols during aerobic pretreatment (Garcia et al., 1997). The fungi conformed to the Monod equation when grown with COD as the substrate. The maximum specific growth rate and kinetic constant were 0.06 h^{-1} and 13525 mg COD/l for *Aspergillus niger* and 0.047 h^{-1} and 4558 mg COD/l for *Geotrichum candidum*. The cell yields relative to the substrate were 0.38 mg cells/mg COD for *Aspergillus terreus* and 0.39 mg cells/mg COD for *G. candidum*. The efficiencies to degrade the phenolic compounds by *A. terreus* and *G. candidum* were 0.033 and 0.036 mg total phenol/mg cell, respectively. The kinetic model showed slightly better behavior for *G. candidum*.

The growth kinetics of *Saccharomyces cerevisiae* using sugarcane molasses has been studied by the substrate inhibition model (Win et al., 1996). Associated parameters of a fermentation model for fed-batch culture and the optimum level of reducing sugar have been determined using nonlinear regression. A feeding procedure to control reducing sugar at the optimum level has been developed. Data from fed-batch experiments have been used to determine the optimum reducing sugar concentration and optimum specific growth rate of *S. cerevisiae*. The feed rate calculated by an equation was corrected by testing the reducing sugar concentration at sampling time and reducing an excess of reducing sugar to the optimum level, resulting in higher

productivity and cell yield. The experimental results showed increased productivity and overall cell yield by controlling sugar at an optimum level, which was determined by the fermentation model. Agrawal et al. (1989) used an algorithm for an online estimate of a fed-batch fermentation rate without measuring nutrient concentration.

A mathematical model, kinetics of biodegradation, and determination of the macroenergetic parameters for the anaerobic digestion of molasses with aerobic pretreatment by *Penicillium decumbens* have been described (Jimenez et al., 1997). A favorable effect of the aerobic pretreatment was shown on the kinetics of the anaerobic digestion process of molasses. The experimental data were used in an equation from which the kinetic constant was calculated. The overall kinetic constant value for pretreated molasses was approximately double that for untreated molasses. This is due to the fact that treatment with *P. decumbens* removed 67.7% of the phenols present in the molasses. A mathematical model using three differential equations was formulated for the aerobic degradation of vinasses with *P. decumbens*, assuming that a fraction of the substrate is partially biodegradable (Jimenez et al., 2005). The model accurately predicted biomass concentrations with time in the process and parameters that affect the aerobic degradation.

The kinetic behavior and flocculation characteristics of *Candida rugosa* at 20, 30, and 40°C in a batch fermentation of sugar beet stillage have been studied (Lee and Baerwald, 1991). The batch fermentation kinetics on 8% dry matter of sugarcane stillage was examined during the growth of *Candida rugosa* at 40°C (Lee and Lee, 1995). The specific growth rate of *C. rugosa* is $0.17\,h^{-1}$ and productivity of biomass is 0.91 g/l per hour. These values are somewhat lower than those of sugar beet stillages. During assimilation, organic acids and glycerol were consumed rapidly and the COD reduction of sugarcane stillage remained 41%.

3.11   ECONOMIC IMPORTANCE

Fungi produce a double benefit during the bioconversion of distillery and brewery wastes. Effluent is purified concomitant with the production of fungal biomass rich in protein. The bioconversion of different stillage by strains of *Candida utilis*, various other yeasts, and filamentous fungi is well established (Friedrich et al., 1992; Wilkie et al., 2000).

3.11.1   Single-Cell Protein Production

The scarcity of protein-rich food has forced humankind to explore alternative sources of protein. Single-cell proteins (SCPs) in different regions of the world have recently been reviewed (Anupama and Ravindra, 2000). Singh (1998) discussed the SCP production of *Saccharomyces cerevisiae* on molasses stillage. SCPs on malt whiskey stillage can also be used as an aquaculture

feed (Murray and Marchant, 1986). A mixed culture of *Candida krusei*, *Geotrichum candidum*, and *Hansenula anomala* was used to replace up to 50% of the casein protein in the feed of rainbow trout. Martin et al. (1993) reported a *C. utilis* biomass exhibiting a good amino acid composition with a protein content of 520 g/kg and a relatively low lipid content. Dry yeast is a suitable source of protein in the diet of cultivated rainbow trout.

Lee and Kim (2001) produced *C. utilis* biomass as aquafeeds by three different types of aerobic fermentations on molasses. The biomass yield and productivity were 0.67 and 0.24 for a batch culture, 0.51 and 1.95 for a fed-batch culture with sigmoidal feeding strategy, and 0.36 g/g and 2.15 g/l per hour for continuous cultures, respectively. The fed-batch fermentation was best for mass production of *C. utilis* and calculated to be $2.76 per kilogram of dry cells. The favorable production cost matched well with the sale price of the commercially yeast product. Highly concentrated Brazilian sugarcane stillage favored a biomass production of 20.4 g/l using the thermotolerant yeast *Candida rugosa* (Lee and Lee, 1995). Shojaosadati et al. (1999) obtained 39.6% crude protein content in *Hansenula* sp. in the absence of additives and 50.6% in the presence of additives such as nitrogen and phosphorus sources. The amount of essential amino acid profiles of SCP is greater than the Food and Agriculture Organization (FAO) reference protein and is comparable to that of some other proteins, such as soybean and fish meal.

Aspergillus awamori var. *kawachi* produced a yield of 40 g/l of biomass, 40% protein content, and 44% reduction of TOC in jar-fermentor culture in *Shochu* distillery wastewater (Morimura et al., 1994b). Digestibility of the biomass was determined by digestion with pepsin and was about 52% without pretreatment, increasing to 62% after heating at 100°C for 20 minutes. Higher yield and composition of biomass within 24 hours indicate its use as a fodder.

Selenium in the organic bound is accepted as an essential element for the growth of animals and humans. Nagodawithana and Gutmanis (1985) revealed organically bound selenium yeast production with 1000 µg of selenium per gram of dry biomass using sodium selenite as the selenium source. Production of organically bound selenium was enhanced in a fed-batch fermentation with continuous feeding of sodium selenite using *Saccharomyces cerevisiae* (Demirci et al., 1999). Two adapted strains, FD10 and FD14, displayed the highest selenium incorporation, 3200 µg per gram of dry biomass.

3.11.2 Ethanol Production

Ethanol is one of the major bioenergy sources that can be produced from sugarcane molasses using strains of *S. cerevisiae* through batch fermentation processes. During the last two decades, several approaches to increased productivity of ethanol have been used, such as yeast recycling, strain selection, and fed-batch and continuous fermentation. Ethanol production from distillery and brewery wastes by fungi is noted in Table 3.5.

TABLE 3.5 Ethanol Production from Distillery and Brewery Wastes

Fungus	Stillage Origin	Bioreactor Type	Ethanol Production (%)	Duration (hours/days)	Reference
Saccharomyces cerevisiae F111	Sugarcane molasses	Fermentation vessel, 87.5 m^3	7.7	27.5h	Abdel-Fattah et al., 2000
Kluyveromyces marxianus WR12			7.4	27.8h	
S. cerevisiae HAU-1	Sugarcane molasses	Immobilized Ca–alginate column reactor	7.0	10d	Sheoran et al., 1998
S. cerevisiae	Sugarcane molasses	Agar immobilized tubular reactor	9.5	100d	Nigam et al., 1998
K. marxianus IMB3 var. *marxianus*	Molasses	Shake flasks	7.4	16–24h	Gough et al., 1996
S. cerevisiae	Beet molasses	Immobilized Ca–alginate fermentor	5.3	24h	Roukas, 1995

3.11.3 Bioproducts

In recent years, the use of stillage in the production of various biological products, such as enzymes, plant hormones, chitosan, astaxanthin, and the biopolymers alternan and pullulan, has received considerable attention (Table 3.6). In one report, the production of gibberellic acid (GA_3) by *Aspergillus niger* in media prepared from molasses and vinasse was 310 and 273 mg/l, respectively (Cihangir and Aksoz, 1996). Yurekli et al. (1999) demonstrated the production of indole acetic acid, gibberellic acid, abscisic acid, and cytokinin by both *Funalia trogii* and *Trametes versicolor* at a 1:4 dilution of stillage. Chitosan has been produced by *Gongronella butleri* on *Shochu* distillery wastewater and resulted in a 49% COD reduction (Yokoi et al., 1998). *Lentinus* (*Lentinula*) *edodes* produced high levels of manganese peroxidase (MnP) and laccase on a malt-containing by-product of the brewing process when mixed with oak wood chips (Hatvani and Mecs, 2001).

Astaxanthin, a highly oxygenated carotenoid produced by *Phaffia rhodozyma*, has two main uses in aquaculture and orthomolecular medicine (Fontana et al., 1997). Low biomass yield and intermediate astaxanthin content have been obtained in 1:10 diluted crude sugarcane juice, but these were enhanced by the addition of corn steep liquor. Roukas and Liakopoulou-Kyriakides (1999) investigated the production of pullulan from treated beet molasses by *Aureobasidium pullulans* in a stirred-tank fermentor, as well as the effect of various fermentation conditions. An aeration rate of 1.0 (v/v) per minute resulted in a pullulan concentration of 23 g/l and a pullulan yield of 40%. The addition of phytate to untreated beet molasses enhanced the production of citric acid by *Aspergillus niger* (Jianlong, 1998). *Aspergillus terreus* secreted riboflavin (vitamin B_2) in a medium containing beet molasses as a sole source of carbon (Sabry et al., 1993). Sterols were produced by some molds in beet molasses–containing medium (Ghanem et al., 1990). For other molds, *Penicillium crustosum* exhibited a superior ability for a total sterol production of 4% on a dry weight basis. Maximum fermentation yields occurred when beet molasses was treated with H_2SO_4 followed by centrifugation. Maximum sterol yields of 8.4% were produced in a medium at pH 7.0 after 8 days of fermentation. Balobova and Kondrashchenko (2000) examined the composition and properties of molasses-containing yeast fermentation waste as a plasticizer and biological additive to concrete mixtures.

Glycerol has an important production history and is used in several processes, such as the manufacture of drugs, cosmetics, toothpaste, lubricants, tobacco processing, and food production. Glycerol can be produced from alcoholic fermentation of sugarcane molasses using *Saccharomyces cerevisiae* (Mostafa, 1995). The maximum glycerol production was obtained on molasses (24.35%) after 48 hours. Mostafa and Magdy (1998) examined a continuous fermentation process for glycerol production from crude untreated sugarcane molasses in a vertical packed-bed reactor with *S. cerevisiae* cells immobilized in agar gel. A higher dilution rate of $0.56 h^{-1}$ from molasses

TABLE 3.6 Formation of Bioproducts During Bioconversion of Distillery and Brewery Wastes

Fungus	Stillage Origin	Bioproduct	Reference
Phaffia rhodozyma	Crude sugarcane juice	Astaxanthin	Fontana et al., 1997
Leuconostoc mesenteroides	Fuel ethanol residues	Alternan	Leathers, 1998
Aureobasidium sp. strain NRRL Y-12974	Fuel ethanol by-products	Pullulan	Leathers and Gupta, 1994
Aureobasidium pullulans	Beet molasses	Pullulan	Roukas and Liakopoulou-Kyriakides, 1999
Gongronella butleri	*Shochu* distillery wastewater	Chitosan	Yokoi et al., 1998
Lentinus (Lentinula) edodes	Malt-containing by-product	MnP and laccase	Hatvani and Mecs, 2001
Aspergillus usami mut. *shirousami*	*Shochu* stillage	Protease	Morimura et al., 1994a
Funalia trogii, Trametes versicolor	Alcohol factory stillage	Indole acetic acid, abscisic acid, gibberellic acid, and cytokinin	Yurekli et al., 1999
Aspergillus awamori var. *kawachi*	*Shochu* stillage	Fodder and α-amylase	Morimura et al., 1991; Morimura et al., 1994b; Kida et al., 1995
Aspergillus niger	Untreated beet molasses	Citric acid	Jianlong, 1998
Aspergillus terreus	Beet molasses	Riboflavin	Sabry et al., 1993
Saccharomyces cerevisiae	Sugarcane molasses	Glycerol	Mostafa, 1995; Mostafa and Magdy, 1998
Molds, especially *Penicillium crustosum*	Beet molasses–containing medium	Sterols	Ghanem et al., 1990

yielded the maximum glycerol production (5.3%) and maximum productivity (29.7 g/l per hour) after 3 hours.

3.11.4   Algal Production

Good information is available on the pollution deloading of distillery wastes by fungi, but little is known of the removal of nutrients such as nitrogen and phosphorus by algae in these effluents. Utilization of these effluents for the preparation of yeast or fungal inoculum has also been recognized. Ferraz et al. (1986) reported on the utilization of dilute molasses stillage along with 74% elimination of COD by *Spirulina maxima*. However, *Spirulina platensis* is known for utilizing nutrients from effluents, is easy to harvest, and has a potential market as an animal feed supplement. Kadioglu and Algur (1992) found that the growth of *Chlamydomonas reinhardii* was stimulated by the addition of 5% vinasse, and a reduction in growth with over 10% stillage. Some microalgae have a potential for the removal of heavy metals from distillery efflents (Travieso et al., 1999a). Travieso et al. (1999b) reported on nutrient removal and potential growth measurement in distillery wastes by *Chlorella vulgaris*.

3.12   BIOTECHNOLOGY

Screening of a number of strains and optimization of the respective fermentations are important for the production of high biomass yield. Several studies related to such screening have been reported in the literature during the last two decades. The optimum temperature ranges between 25 and 30°C for most strains of *S. cerevisiae*. A few reports discuss the growth and ethanol fermentation of *S. cerevisiae* at higher temperatures: 40°C (Banat et al., 1998), with *S. cerevisiae* F111 at 43°C (Abdel-Fattah et al., 2000). Several reports have appeared on thermotolerant strains of *Kluyveromyces* (Banat et al., 1992; Banat and Marchant, 1995; Singh et al., 1998b; Abdel-Fattah et al., 2000). Two reports are known concerning the use of thermotolerant yeasts in regular industrial ethanol production: *Kluyveromyces marxianus* IMB3 (Singh et al., 1998a) and *S. cerevisiae* F111 (Abdel-Fattah et al., 2000). Wati et al. (1996) isolated and characterized some thermotolerant mutants of haploid strains of *S. cerevisiae* by physical and chemical methods of mutagenesis. Protoplast fusion of two yeast strains has been used to obtain a thermotolerant flocculating strain of *S. cerevisiae* KF-7 (Kida et al., 1992). Further studies on this flocculating strain suggested stability in repeated-batch fermentation (Shou et al., 1995) and ethanol production by repeated-batch fermentation in a molasses medium with improved osmotolerance (Morimura et al., 1997) at 35°C.

Hansenula anomala J 45-N-5, a mutant, is also suitable for the treatment of distillery wastewater (Moriya et al., 1990). Ohta et al. (1988) discussed the

temperature-sensitive mutant of a thermotolerant yeast, *Hansenula polymorpha*. Production of ethanol by *Escherichia coli* carrying genes from *Zymomonas mobilis* has been demonstrated (Lawford and Rousseau, 1991). Additional substrate abilities to utilize pentoses and hexoses for ethanol production have been revealed by genetically engineered *Saccharomyces* (Ho et al., 1998) and strains of *S. cerevisiae* expressing XYL1 and XYL2 from *Pichia stipitis* with or without expression of TAL1 (Meinander et al., 1999). Such strains can also lead to reduction in the COD of stillage during fermentation. Tremendous scope exists for the identification, sequencing, and expression of different genes specific for decolorization of melanoidins or vinasses in various strains of yeasts or fungi. Kanda and Kuwahara (1991) reported on the breeding of higher glycerol-producing mutants from allyl alcohol– and pyrazole-resistant *S. cerevisiae* K-7 and its haploid strain. Respiratory deficiency is noted in all higher glycerol-producing mutants obtained from allyl alcohol–resistant K-7 diploids. However, the other higher glycerol-producing mutants (pyrazole resistant from K-7 diploids and allyl alcohol resistant from K-7 haploids) are not respiratory deficient. These mutants produce more glycerol than K-7 and the same ethyl alcohol as K-7. However, more research is required for the breeding of excellent yeasts for use in distillery wastewater treatment.

Recent advances in molecular microbial ecology have advocated a variety of new tools to assess directly the microbial diversity present in natural habitats (Head et al., 1998) without microbial enrichment. However, the yeasts in ethanol fermentations or in melanoidin decolorization bioreactors have not been well characterized. Cocolin et al. (2000) presented a method to characterize the yeast diversity in wine fermentations by employing denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR)–amplified 26S ribosomal RNA (rRNA) genes. This method can also be used in direct profiling of the yeast dynamics in melanoidin fermentations. PCR-DGGE can assess the populations of yeast diversity in fermentation as low as 1000 cells/ml, thus representing an attractive alternative to traditional plating methods.

3.13   CONCLUSIONS AND FUTURE PERSPECTIVES

The purpose of treatment of variable distillery wastewater is to discharge colorless effluent to receiving water bodies or to dispose of them on land, which ever is acceptable to the public. The decolorization of melanoidin pigments is the major concern. Certain physical, chemical, and thermal methods have been used but are unsuitable in commercial applications. Highly successful and popular stillage treatments use anaerobic digestion to reduce COD, BOD, and methane production, but involve high installation and implementation costs.

Considerable success has been achieved by the use of fungi in the treatment of distillery and brewery wastes. More factors affecting the efficiency of

fungal fermentation, higher decolorization yields, and COD and BOD removal in distillery and brewery effluents are yet to be discovered and optimized. Development of the treatment system must consider dilution of the MSW. Optimizing conditions for melanoidin color removal by immobilized *Phanerochaete chrysosporium* have a potential for bioremediation of MSW wastewater (Fahy et al., 1997). A biological decolorization process with *Flavodon flavus* can be implemented on a large scale in shallow ponds and reactors in the presence of a high oxygen content (Raghukumar and Rivonkar, 2001). Color removal under a wide range of nutrient conditions is an added advantage. Decolorization of MSW using this fungus can also be integrated into the effluent treatment system prior to primary and secondary treatments. Fungal treatment reduces COD levels, and further reduction in COD can be achieved during primary and secondary treatments. A combination of treatment processes can result in effective mycoremediation of highly recalcitrant MSW wastewaters.

Various fungal bioreactors for stillage treatment were discussed in the foregoing paragraphs. Cheaper and natural substrate sources have been considered and can be substituted for synthetic and expensive sources. A process should be devised to extend biomass activity for a longer period, to minimize nutrients, and to handle a large volume of unsterilized spent wash. A shorter cultivation period, an easy method of cultivation, and minimum precautions against bacterial contamination must also be taken into account in the feasibility of a continuous system. All these conditions can add knowledge toward a realistic approach to treatment without additional cost. By optimizing these conditions, suitable and efficient bioreactor configurations to exploit the bioremediation capacities of fungi along with prolonged operational stabilities can be developed, resulting in a high rate of melanoidin degradation and decolorization efficiency.

Scanty reports exist on the role of certain enzymes for melanoidin decolorization, and a complete enzymatic system for the decolorization of melanoidin is far from established. In certain cases, a certain percentage of color adsorption to fungal mycelia has been documented. A new horizon on the role of specific mediators or inducers related to the enhancement of enzyme activity for melanoidin decolorization can be opened. Little is known as to the mechanism of action of melanoidin degradation, and further studies on this subject are required. Modeling studies on molasses decolorization are also few. Moreover, methods to reduce toxicity of molasses can open doors to innovative treatments. The lack of general availability of this knowledge is limiting commercial applications of fungal treatments.

Fungal treatment of distillery and brewery wastewater is of great significance because this procedure produces a valuable by-product along with molasses decolorization and removal of COD and BOD. This by-product is a single-cell protein (SCP), especially important for countries that have a scarcity of protein resources. The other process of economic importance is that yeasts utilize toxic and inhibitory components, such as acetate and lactate,

resulting in ethanol production, and consequently, the effluent can be recycled as diluting water in a fermentor (Shojaosadati et al., 1999). This process reduces water consumption for dilution of molasses and the volume of residual stillage by 70% and offers more economic benefits for further treatment. The profits due to annual ethanol production capacity are increased significantly, while the need for cooling water is eliminated.

REFERENCES

Abdel-Fattah, W.R., M. Fadil, P. Nigam, and I.M. Banat (2000) Isolation of thermotolerant ethanologenic yeasts and use of selected strains in industrial scale fermentation in an Egyptian distillery. *Biotechnol. Bioeng.* **68**: 531–535.

Agrawal, P., G. Koshy, and M. Ramseier (1989) An algorithm for operating a fed-batch fermenter at optimum specific-growth rate. *Biotechnol. Bioeng.* **33**: 115–125.

Akaki, M., T. Takahashi, and K. Ishiguro (1981) Studies on microbiological treatment and utilization of cane molasses distillery wastes. I. Screening of useful yeast strains. *Mie Daigaku Nogakubu Gakujutsu Hokoku* **62**: 155–161.

Akunna, J.C., and M. Clark (2000) Performance of a granular-bed anaerobic baffled reactor (GRABBR) treating whisky distillery wastewater. *Bioresour. Technol.* **74**: 257–261.

Algur, O.F., and H.Y. Gokalp (1991) Batch culture of *Rhizopus arrhizus* and *Actinomucor elegans* on vinasse medium for biomass production and BOD reduction. *Doga Turk. J. Biol.* **15**: 198–205.

Anupama, and P. Ravindra (2000) Value-added food: single cell protein. *Biotechnol. Adv.* **18**: 459–479.

Aoshima, I., Y. Tozawa, S. Ohmomo, and K. Ueda (1985) Production of decolorizing activity for molasses pigment by *Coriolus versicolor* Ps4a. *Agric. Biol. Chem.* **49**: 2041–2045.

Arasaratnam, V., and K. Balasubramaniam (1998) The use of monochloroacetic acid for improved ethanol production by immobilized *Saccharomyces cerevisiae. World J. Microbiol. Biotechnol.* **14**: 107–111.

ASTM (1997) Standard guide for evaluation of fuel ethanol manufacturing facilities. E1344-90. In: *Annual Book of ASTM Standards, Biological Effects and Environmental Fate; Biotechnology; Pesticides.* American Society for Testing and Materials, West Conshohocken, PA, Vol. 11.05, pp. 667–689.

Baerwald, G., and K.Y. Lee (1989) über die Zusammensetzung von Reubenmelasseschlempe hinsichtlich ihrer Verhefung. *Branntweinwirtschaft* **129**: 146–152.

Balaguer, M.D., M.Y. Vincent, and J.M. Paris (1992) Anaerobic fluidized bed reactor with sepiolite as support for anaerobic treatment of vinasse. *Biotechnol. Lett.* **14**: 433–438.

Balobova, A.V., and V.I. Kondrashchenko (2000) Fermentation waste: a biomodifier of concrete. *Prikl. Biokhim. Mikrobiol.* **36**: 243–253.

Banat, I.M., and R. Marchant (1995) Characterization and potential industrial applications of five novel, thermotolerant, fermentative yeast strains. *World J. Microbiol. Biotechnol.* **11**: 304–306.

Banat, I.M., P. Nigam, and R. Marchant (1992) Isolation of thermotolerant, fermentative yeasts growing at 52°C and producing ethanol at 45°C and 50°C. *World J. Microbiol. Biotechnol.* **8**: 259–263.

Banat, I.M., P. Nigam, D. Singh, R. Marchant, and A. McHale (1998) Ethanol production at elevated temperatures and alcohol concentrations. I. Yeast in general. *World J. Microbiol. Biotechnol.* **12**: 809–821.

Barker, T.W., J.P. Quinn, and R. Marchant (1982) The use of a mixed culture of *Geotrichum candidum*, *Candida krusei* and *Hansenula anomala* for microbial protein production from whisky distillery spent wash. *Eur. J. Appl. Microbiol. Biotechnol*. **14**: 247–253.

Belkacemi, K., F. Larachi, S. Hamoudi, and A. Sayari (2000) Catalytic wet oxidation of high-strength alcohol-distillery liquors. *Appl. Catal. A* **199**: 199–209.

Beltran, F.J., J.F. Garcia-Araya, and P.M. Alverez (1999a) Wine distillery wastewater degradation. 1. Oxidative treatment using ozone and its effect on the wastewater biodegradability. *J. Agric. Food Chem.* **47**: 3911–3918.

Beltran, F.J., J.F. Garcia-Araya, and P.M. Alverez (1999b) Wine distillery wastewater degradation. 2. Improvement of aerobic degradation by means of an integrated chemical (ozone)–biological treatment. *J. Agric. Food Chem*. **47**: 3919–3924.

Benito, G.G., M.P. Miranda, and D.R. Santos (1997) Decolorization of wastewater from an alcoholic fermentation process with *Trametes versicolor. Bioresour. Technol.* **61**: 33–37.

Buffiere, P., J.-P. Bergeon, and R. Moletta (2000) The inverse turbulent bed: a novel bioreactor or anaerobic treatment. *Water Res.* **34**: 673–677.

Cabib, G., H.J. Silva, A. Giulietti, and R. Ertola (1983) The use of sugar cane stillage for single cell protein production. *J. Chem. Tech. Biotechnol. B* **33**: 21–28.

Castlla, R.B., R.S. Waehner, and A.M. Giulietti (1984) Aerobic microbial treatment of sugar cane stillage by *Candida utilis* and *Paecilomyces variotii* in two step continuous cultures. *Biotechnol. Lett*. **6**: 195–198.

Ciftci, T., and I. Ozturk (1995) Nine years of full-scale anaerobic–aerobic treatment experiences with fermentation industry effluents. *Water Sci. Technol*. **32**: 131–139.

Cihangir, N., and N. Aksoz (1996) Production of gibberellic acid by *Aspergillus niger* using some food industry wastes. *Acta Microbiol. Pol.* **45**: 291–297.

Cocolin, L., L.F. Bisson, and D.A. Mills (2000) Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiol. Lett.* **189**: 81–87.

Dahiya, J., D. Singh, and P. Nigam (2001) Decolourization of synthetic and spentwash melanoidins using the white-rot fungus *Phanerochaete chrysosporium* JAG-40. *Bioresour. Technol.* **78**: 95–98.

Dehorter, B., and R. Blondeau (1993) Isolation of an extracellular Mn-dependent enzyme mineralizing melanoidins from the white rot fungus *Trametes versicolor. FEMS Microbiol Lett.* **109**: 117–122.

de Lamo, P.R., and T.J.B. de Menezes (1978) Bioconversao da vinhaca para producao de biomassa fungica. *Coletanea Inst. Tecnol. Alimentos* **9**: 281–312.

de Menezes, T.J.B. (1989) The treatment and utilization of alcohol stillage. In: *International Biosystems*, D.L. Wise, ed. CRC Press, Boca Raton, FL, Vol. III, pp. 1–14.

Demirci, A., A.L. Pometto III, and D.J. Cox (1999) Enhanced organically bound selenium yeast production by fed-batch fermentation. *J. Agric. Food Chem.* **47**: 3496–3500.

DNDE (1998) *BEB 98 Electronic Version: Brazilian Energy Balance—1982 to 1997 Period*. National Department of Energy Development of the Secretariat of Energy of the Ministry of Mines and Energy (DNDE/SEN/MME), Brasilia, Brazil.

Durrant, L.R. (1996) Decolorization of sugar cane molasses by ligninolytic cultures of *Phanerochaete chrysosporium*. *Int. Biodeterior. Biodegrad.* **37**: 113.

Fahy, V., F.J. FitzGibbon, G. McMullan, D. Singh, and R. Marchant (1997) Decolourization of molasses spent wash by *Phanerochaete chrysosporium*. *Biotechnol. Lett.* **19**: 97–99.

Ferraz, C.A.M., E. Aquarone, and M. Krauter (1986) Utilization of by-products from alcoholic fermentation industry to biomass production of *Spirulina maxima*. II. Use of molasses alcohol distillate waste. *Rev. Microbiol.* **17**: 15–21.

FitzGibbon, F.J., P. Nigam, D. Singh, and R. Marchant (1995) Biological treatment of distillery waste for pollution-remediation. *J. Basic Microbiol.* **35**: 293–301.

FitzGibbon, F.J., D. Singh, G. McMullan, and R. Marchant (1998) The effect of phenolic acids and molasses spent wash concentration on distillery wastewater remediation by fungi. *Process Biochem.* **33**: 799–803.

Fontana, J.D., M.B. Chocial, M. Baron, M.F. Guimaraes, M. Maraschin, C. Ulhoa, Florencio, J.A., and T.M. Bonfim (1997) Astaxanthinogenesis in the yeast *Phaffia rhodozyma*: optimization of low-cost culture media and yeast cell-wall lysis. *Appl. Biochem. Biotechnol.* **63–65**: 305–314.

Friedrich, J., A. Cimerman, and A. Perdih (1986) Comparison of different cellulolytic fungi for bioconversion of apple distillery waste. *Appl. Microbiol. Biotechnol.* **24**: 432–434.

Friedrich, J., A. Cimerman, and A. Perdih (1992) Use of fungi for bioconversion of distillery waste. In: *Handbook of Applied Mycology*, D.K. Arora, R.P. Elander, and K.G. Mukerji, eds. Marcel Dekker, New York, Vol. 4, pp. 963–992.

Gabara, B., and Z. Zakowska (1997) Effect of Spumol K on cell wall of *Aspergillus niger* strains characterized by different tolerance to toxic compounds of beet molasses. *Acta Microbiol. Pol.* **46**: 219–223.

Garcia, I.G., J.L.B. Venceslada, P.R.J. Pena, and E.R. Gomez (1997) Biodegradation of phenol compounds in vinasse using *Aspergillus terreus* and *Geotrichum candidum*. *Water Res.* **31**: 2005–2011.

Ghanem, K.M., N.B. Ghanem, and A.H. el-Refai (1990) Utilization of beet molasses for sterol production by some moulds. *Microbiologia* (*Madrid*) **6**: 37–44.

Gonzalez, T., M.C. Terron, S. Vague, E. Zapico, G.C. Galletti, and A.E. Gonzalez (2000) Pyrolysis/gas chromatography/mass spectrometry monitoring of fungal-biotreated distillery wastewater using *Trametes* sp. I-62 (CECT 20197). *Rapid Commun. Mass Spectrom.* **14**: 1417–1424.

Goto, M., T. Nada, A. Kodama, and T. Hirose (1999) Kinetic analysis for destruction of municipal sewage sludge and alcohol distillery wastewater by supercritical water oxidation. *Ind. Eng. Chem. Res.* **38**: 1863–1865.

Gough, S., O. Flynn, C.J. Hack, and R. Marchant (1996) Fermentation of molasses using a thermotolerant yeast, *Kluyveromyces marxianus* IMB3: simplex optimisation of media supplements. *Appl. Microbiol. Biotechnol.* **46**: 187–190.

Guimaraes, C., L.S.M. Bento, and M. Mota (1996) Assessment of the decolourization ability of *Phanerochaete chrysosporium* towards different sugar colourants. In: *Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry: Recent Advances in Applied and Fundamental Research*, E. Srebotnik and K. Messner, eds. Facultas-Universitätsverlag, Vienna, Austria, pp. 259–262.

Guruswami, R. (1988) Pollution control in distillery industry. In: *National Seminar on Pollution Control in Sugar and Allied Industries*, Bombay, India.

Hanqing, Y., and F. Guowei (1996) Biomethanation of brewery wastewater using an anaerobic upflow blanket filter. *J. Clean. Prod.* **4**: 219–223.

Harada, H., S. Uemura, A.-C. Chen, and J. Jayadevan (1996) Anaerobic treatment of a recalcitrant wastewater by a thermophilic UASB reactor. *Bioresour. Technol.* **55**: 215–221.

Hatvani, N., and I. Mecs (2001) Production of laccase and manganese peroxidase by *Lentinus edodes* on malt-containing by-product of the brewing process. *Process Biochem.* **37**: 491–496.

Head, I.M., J.R. Saunders, and R.W. Pickup (1998) Microbial evolution, diversity and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms *Microb. Ecol.* **35**: 1–21.

Henry, D.P., and R.H. Thomson (1993) A new process to treat strong biological waste. *Water Sci. Technol.* **27**: 213–218.

Hickey, R.F., W.M. Wu, M.C. Veiga, and R. Jones (1991) Start-up, operation, monitoring and control of high-rate anaerobic treatment systems. *Water Sci. Technol.* **24**: 207–255.

Ho, N.W.Y., Z. Chen, and A.P. Brainard (1998) Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. *Appl. Environ. Microbiol.* **64**: 1852–1859.

Ince, B.K., O. Ince, J. Sallis, and S.K. Anderson (2000) Inert COD production in a membrane anaerobic reactor treating brewery wastewater. *Water Res.* **34**: 3943–3948.

Ingledew, W.M. (1995) The biochemistry of alcohol production. In: *The Alcohol Textbook*, T.P. Lyons, D.R. Kelsall, and J.E. Murtagh, eds. Nottingham University Press, Nottingham, UK, pp. 55–79.

Jafelice, L.R., A. Wiseman, and P.S. Goldfarb (1990) Production of lignin peroxidase by *Phanerochaete chrysosporium* in medium containing molasses. *Biochem. Soc. Trans.* **18**: 642–643.

Jianlong, W. (1998) Improvement of citric acid production by *Aspergillus niger* with addition of phytate to beet molasses. *Bioresour. Technol.* **65**: 243–245.

Jimenez, A.M., R. Borja, V. Alonso, and A. Martin (1997) Influence of aerobic pretreatment with *Penicillium decumbens* on the anaerobic digestion of beet molasses alcohol fermentation wastewater in suspended and immobilized cell bioreactors. *J. Chem. Tech. Biotechnol.* **69**: 193–202.

Jimenez, A.M., R. Borja, and A. Martin (2003) Aerobic–anaerobic biodegradation of beet molasses alcoholic fermentation wastewater. *Process Biochem.* **38**: 1275–1284.

Jimenez, A.M., R. Borja, A. Martin, and F. Raposo (2005) Mathematical modeling of aerobic degradation of vinasses with *Penicillium decumbens. Process Biochem.* **40**: 2805–2811.

Kadioglu, A., and O.F. Algur (1992) Tests of media with vinasse for *Chlamydomonas reinhardii* for possible reduction in vinasse pollution. *Bioresour. Technol.* **42**: 1–5.

Kanda, A.H., and M. Kuwahara (1991) *Hakko Kogaku Kaishi.* General Research Institute of the Ozeki Sake Brewing Co. Ltd., Nishinoniya, Japan, pp. 203–209.

Kato, M.T., S. Rebac, and G. Lettinga (1999) Anaerobic treatment of low-strength brewery wastewater in expanded granular sludge bed reactor. *Appl. Biochem. Biotechnol.* **76**: 15–32.

Kida, K., K. Kume, S. Morimura, and Y. Sonoda (1992) Repeated-batch fermentation process using a thermotolerant flocculating yeast constructed by protoplast fusion. *J. Ferment. Biotechnol.* **74**: 169–173.

Kida, K., S. Morimura, N. Abe, and Y. Sonoda (1995) Biological treatment of *shochu* distillery wastewater. *Process Biochem.* **30**: 125–132.

Kim, S.J., and M. Shoda (1999) Decolorization of molasses and a dye by a newly isolated strain of the fungus *Geotrichum candidum* Dec 1. *Biotechnol. Bioeng.* **62**: 114–119.

Kim, J.-S., B.-G. Kim, and C.-H. Lee (1999) Distillery waste recycle through membrane filtration in batch alcohol fermentation. *Biotechnol. Lett.* **21**: 401–405.

Kumar, S., S.S. Sahay, and M.K. Sinha (1995) Bioassay of distillery effluent on common guppy, *Lebistes reticulatus* (Peter). *Bull. Environ. Contam. Toxicol.* **54**: 309–316.

Kumar, V., L. Wati, P. Nigam, I.M. Banat, B.S. Yadav, D. Singh, and R. Marchant (1998) Decolorization and biodegradation of anaerobically digested sugar cane molasses spent wash effluent from biomethanation plants by white-rot fungi. *Process Biochem.* **33**: 83–88.

Lalov, I.G., I.I. Guerginov, M.A. Krysteva, and K. Fartsov (2000) Treatment of waste waters from distilleries with chitosan. *Water Res.* **34**: 1503–1506.

Lawford, H.G., and J.D. Rousseau (1991) Ethanol production by recombinant *Escherichia coli* carrying genes from *Zymomonas mobilis. Appl. Biochem. Biotechnol.* **28–29**: 221–236.

Leal, K., E. Chacin, E. Behling, E. Gutierez, N. Fernandez, and C.F. Forster (1998) A mesophilic digestion of brewery wastewater in an unheated anaerobic filter. *Bioresour. Technol.* **65**: 51–55.

Leathers, T.D. (1998) Utilization of fuel ethanol residues in production of the biopolymer alternan. *Process Biochem.* **33**: 15–19.

Leathers, T.D., and S.C. Gupta (1994) Production of pullulan from fuel ethanol byproducts by *Aureobasidium* sp. strain NRRL Y-12,974. *Biotechnol. Lett.* **16**: 1163–1166.

Lee, K.Y. (1991) The growth, effect of COD-reduction, and flocculation characteristics of *Candida rugosa* in sugar beet stillages. *J. Microbiol. Biotechnol.* **1**: 207–211.

Lee, K.Y., and G. Baerwald (1991) Kinetic behavior of *Candida rugosa* in the batch fermentation of sugar beet stillage: temperature dependance of growth and flocculation characteristics. *Biotechnol. Lett*. **13**: 595–598.

Lee, B.-K., and J.K. Kim (2001) Production of *Candida utilis* biomass on molasses in different culture types. *Aquacult. Eng*. **25**: 111–124.

Lee, K.-Y., and S.-T. Lee (1995) Yeast biomass production from concentrated sugar cane stillage using a thermotolerant *Candida rugosa*. *J. Microbiol. Biotechnol*. **5**: 114–116.

Lele, S.S., P.J. Rajadhyaksha, and J.B. Joshi (1989) Effluent treatment for alcohol distillery: thermal pretreatment with energy recovery. *Environ. Prog*. **8**: 245–252.

Lloyd, D., S. Morell, H.N. Carlsen, et al. (1993) Effects of growth with ethanol on fermentation and membrane fluidity of *Saccharomyces cerevisiae*. *Yeast* **9**: 825–833.

Lynd, L.R., H.-J, Ahn, G. Anderson, P. Hill, D.S. Kersey, and T. Klapatch (1991) Thermophilic ethanol production: investigation of ethanol yield and tolerance in continuous culture. *Appl. Biochem. Biotechnol*. **28**–**29**: 549–570.

Maestro-Duran, R., R. Borja-Padilla, M. Luque-Gonzalez, and A. Martin-Martin (1993) Compuestos fenolicos en las aguas residuales de las destilerías vínicas (vinazas) [Phenolic compounds in waste water from wine distilleries) (vinasse)]. *Rev. Esp. Ciencia Tecnol. Alimentos* **35**: 517–528.

Malandra, L., G. Wolfaardt, A. Zietsman, and M. Viljoen-Bloom (2003) Microbiology of a biological contactor for winery wastewater treatment. *Water Res*. **37**: 4125–4134.

Malnou, D., A. Huyard, and G.M. Faup (1987) High load process using yeasts for vinasses of beet molasses treatment. *Water Sci. Technol*. **19**(Rio): 11–21.

Mansur, M., T. Suarez, J. Fernandez-Larrea, M.A. Brizuela, and A.E. Gonzalez (1997) Identification of a laccase gene family in the new lignin-degrading basidiomycete CECT 20197. *Appl. Environ. Microbiol*. **63**: 2637–2646.

Martin, A.M., S. Goddard, and P. Bemister (1993) Production of *Candida utilis* biomass as aquaculture feed. *J. Sci. Food Agric*. **61**: 363–370.

Meinander, N.Q., I. Boels, and B. Hahn-Hagerdal (1999) Fermentation of xylose/glucose mixtures by metabolically engineered *Saccharomyces cerevisiae* strains expressing *XYL1* and *XYL2* from *Pichia stipitis* with and without overexpression of *TAL1*. *Bioresour. Technol*. **68**: 79–87.

Miranda, M.P., G.G. Benito, N.S. Cristobal, and C.H. Nieto (1996) Color elimination from molasses wastewater by *Aspergillus niger*. *Bioresour. Technol*. **57**: 229–235.

Miyata, N., K. Iwahori, and M. Fujita (1998) Manganese-independent and -dependent decolorization of melanoidin by extracellular hydrogen peroxide and peroxidases from *Coriolus hirsutus* pellets. *J. Ferment. Bioeng*. **85**: 550–553.

Morimura, S., K. Kida, Y. Yakita, Y. Sonoda, and H. Myoga (1991) Production of saccharifying enzyme using the wastewater of a *shochu* distillery. *J. Ferment. Bioeng*. **71**: 329–334.

Morimura, S., K. Kida, and Y. Sonoda (1994a) Production of protease using wastewater from the manufacture of *shochu*. *J. Ferment. Bioeng*. **77**: 183–187.

Morimura, S., K. Kida, M. Nakagawa, and Y. Sonoda (1994b) Production of fungal protein by *Aspergillus awamori* var. *kawachi* grown in *shochu* distillery wastewater. *J. Ferment. Bioeng.* **78**: 160–163.

Morimura, S., Z.Y. Ling, and K. Kida (1997) Ethanol production by repeated-batch fermentation at high temperature in a molasses medium containing a high concentration of total sugar by a thermotolerant flocculating yeast with improved salt-tolerance. *J. Ferment. Bioeng* **83**: 271–274.

Moriya, K., H. Iefuji, H. Shimoi, S.-I. Sato, and M. Tadenuma (1990) Treatment of distillery wastewater discharged from beet molasses-spirits production using yeast. *J. Ferment. Bioeng.* **69**: 138–140.

Mostafa, N.A. (1995) Utilization of molasses and akalona hydrolyzate for glycerol production using a local yeast (*Saccharomyces cerevisiae*). *Biomass Bioenergy* **8**: 427–431.

Mostafa, N.A., and Y.H. Magdy (1998) Utilization of molasses and akalona hydrolyzate for continuous glycerol production in a packed bed bioreactor. *Energy Convers. Manag.* **39**: 671–677.

Murray, A.P., and R. Marchant (1986) Nitrogen utilization in rainbow trout fingerlings (*Salmo gairdneri* Richardson) fed mixed microbial biomass. *Aquaculture* **54**: 263–275.

Nagodawithana, T., and F. Gutmanis (1985) Method for the production of selenium yeast. U.S. patent 4,530,846.

Nigam, J.N., B.K. Gogoi, and R.L. Bezbaruah (1998) Agar immobilized yeast cells in tubular reactor for ethanol production. *Indian J. Exp. Biol*. **36**: 816–819.

Nudel, B.C., R.S, Waehner, E.R. Fraile, and A.M. Giulietti (1987) The use of single and mixed cultures for aerobic treatment of cane sugar stillage and SCP production. *Biol. Wastes* **22**: 67–73.

Ohmomo, S., N. Itoh, Y. Watanabe, Y. Kaneko, Y. Tozawa, and K. Ueda (1985) Continuous decolorization of molasses waste water with mycelia of *Coriolus versicolor* Ps4a. *Agric. Biol. Chem*. **49**: 2551–2555.

Ohmomo, S., Y. Kaneko, S. Sirianuntapiboon, P. Somchai, P. Atthasampunna, and I. Nakamura (1987) Decolorization of molasses waste water by a thermophilic strain, *Aspergillus fumigatus* G-2-6. *Agric. Biol. Chem*. **51**: 3339–3346.

Ohta, K., S.C. Wijeyaratne, and S. Hayashida (1988) Temperature-sensitive mutants of a thermotolerant yeast, *Hansenula polymorpha. J. Ferment. Technol.* **66**: 455–459.

Perdih, A., A. Cimerman, and J. Friedrich (1991) Fungal bioconversion of fruit distillery wastes. *Enzyme Microb. Technol.* **13**: 848–852.

Perez, M., L.I. Romero, and D. Sales (1998) Comparative performance of high rate anaerobic thermophilic technologies treating industrial wastewater. *Water Res.* **32**: 559–564.

Raghukumar, C., and G. Rivonkar (2001) Decolorization of molasses spent wash by the white-rot fungus *Flavodon flavus*, isolated from a marine habitat. *Appl. Microbiol. Biotechnol*. **55**: 510–514.

Raghukumar, C., C. Mohandass, S. Kamat, and M.S. Shailaja (2004) Simultaneous detoxification and decolorization of molasses spent wash by the immobilized white-rot fungus *Flavodon flavus* isolated from a marine habitat. *Enzyme Microb. Technol*. **35**: 197–202.

Rose, A.H. (1993) Composition of the envelope layers of *Saccharomyces cerevisiae* in relation to flocculation and ethanol tolerance. *J. Appl. Bacteriol.* **74**: 110S–118S.

Roukas, T. (1995) Ethanol production from non-sterilized beet molasses by free and immobilized *Saccharomyces cerevisiae* cells using fed-batch culture. *J. Food Eng.* **27**: 87–96.

Roukas, T., and M. Liakopoulou-Kyriakides (1999) Production of pullulan from beet molasses by *Aureobasidium pullulans* in a stirred tank fermentor. *J. Food Eng.* **40**: 89–94.

Ruegger, M.J.S., and S.M. Tauk-Tornisielo (1996) Biomass production by filamentous fungi in sugar cane vinasse medium supplemented with molasses. *Arq. Biol. Tecnol.* **39**: 323–332.

Sabry, S.A., K.M. Ghanem, and H.A. Ghozlan (1993) Riboflavin production by *Aspergillus terreus* from beet-molasses. *Microbiologia* (*Madrid*) **9**: 118–124.

Scheper, T., B. Hitzmann, E. Stark, R. Ulber, R. Faurie, P. Sosnitza, and K.F. Reardon (1999) Bioanalytics: detailed insight into bioprocesses. *Anal. Chim. Acta* **22**: 121–134.

Sheoran, A., B.S. Yadav, P. Nigam, and D. Singh (1998) Continuous ethanol production from sugarcane molasses using a column reactor of immobilized *Saccharomyces cerevisiae* HAU-1. *J. Basic Microbiol.* **38**: 123–128.

Shojaosadati, S.A., H.R. Sanaei, and S.M. Fatemi (1996) The use of biomass and stillage recycle in conventional ethanol fermentation. *J. Chem. Technol. Biotechnol.* **67**: 362–366.

Shojaosadati, S.A., R. Khalilzadeh, A. Jalilzadeh, and H.Z. Sanaei (1999) Bioconversion of molasses stillage to protein as an economic treatment of this effluent. *Resour. Conserv. Recycl.* **27**: 125–138.

Shou, A., S. Morimura, and K. Kida (1995) Effects of aeration and fermentation temperature on stability of repeated-batch fermentation by thermotolerant flocculating yeast. *Seibutsu-Kogaku Kaishi—J. Soc. Ferment. Bioeng.* **73**: 109–112.

Singh, B.D. (1998) *Biotechnology*. Kalyani Publishers, New Delhi, India, pp. 488–510.

Singh, D., and P. Nigam (1995) Treatment and disposal of distillery effluents in India. In: *Environmental Biotechnology: Principles and Application*s, M. Moo-Young et al., eds. Kluwer, Dordrecht, The Netherlands, pp. 735–750.

Singh, D., I.M. Banat, P. Nigam, and R. Marchant (1998a) Industrial scale ethanol production using thermotolerant yeast *Kluyveromyces marxianus* IMB3 in an Indian distillery. *Biotechnol. Lett.* **20**: 753–755.

Singh, D., P. Nigam, I.M. Banat, R. Marchant, and A.P. McHale (1998b) Ethanol production at elevated temperatures and alcohol concentrations: a review. II. Use of *Kluyveromyces marxianus* IMB3. *World J. Microbiol. Biotechnol.* **14**: 823–834.

Sirianuntapiboon, S., P. Somchai, S. Ohmomo, and P. Atthasampunna (1988a) Screening of filamentous fungi having the ability to decolorize molasses pigments. *Agric. Biol. Chem.* **52**: 387–392.

Sirianuntapiboon, S., P. Somchai, P. Sihanonth, P. Atthasampunna, and S. Ohmomo (1988b) Microbial decolorization of molasses waste water by *Mycelia sterilia* D90. *Agric. Biol. Chem.* **52**: 393–398.

Sirianuntapiboon, S., P. Zohsalam, and S. Ohmomo (2004) Decolorization of molasses wastewater by *Citeromyces* sp. WR-43-6. *Process Biochem*. **39**: 917–924.

Suutari, M., K. Liukkonen, and S. Laakso (1990) Temperature adaptation in yeasts: the role of fatty acids. *J. Gen. Microbiol*. **136**: 1469–1474.

Travieso, L., R.O. Canizares, R. Borja, F. Benitez, A.R. Dominguez, et al. (1999a) Heavy metal removal by microalgae. *Bull. Environ. Contam. Toxicol*. **62**: 144–151.

Travieso, L., F. Benitez, and R. Dupeyron (1999b) Algal growth potential measurement in distillery wastes. *Bull. Environ. Contam. Toxicol*. **62**: 483–489.

van Haandel, A.C., and P.F.C. Catunda (1994) Profitability increase of alcohol distilleries by the rational use of byproducts. *Water Sci. Technol*. **29**: 117–124.

van Uden, N. (1984) Temperature profiles of yeasts. In*: Advances in Microbial Physiology*, A.H. Rose and D.W. Tempest, eds. Academic Press, London, pp. 195–248.

Vijayaraghavan, K., T.K. Ramanujam, and N. Balasubramanian (1999) In situ hypochlorous acid generation for the treatment of distillery spentwash. *Ind. Eng. Chem. Res*. **38**: 2264–2267.

Vitali, V.M.V., and C. Kiyan (1996) Bioconversion of sugar cane and citrus vinasse by four *Aspergillus* species. *Int. Biodeterior. Biodegrad*. **37**: 113.

Vlyssides, A.G., C.J. Israilides, M. Loizidou, G. Karvouni, and V. Mourafeti (1997) Electrochemical treatment of vinasse from beet molasses. *Water Sci. Technol*. **36**: 271–278.

Warren, R.K., D.G. Macdonald, and G.A. Hill (1994) The design and costing of a continuous ethanol process using wheat and cell recycle fermentation. *Bioresour. Technol*. **47**: 121–129.

Watanabe, Y., R. Sugi, Y. Tanaka, S. Hayashida (1982) Enzymatic decolorization of melanoidin by *Coriolus* sp. No. 20. *Agric. Biol. Chem*. **42**: 359–367.

Wati, L., S.S. Dhamija, D. Singh, P. Nigam, and R. Marchant (1996) Characterization of genetic control of thermotolerance in mutants of *Saccharomyces cerevisiae*. *Gen. Eng. Biotechnol*. **16**: 19–26.

Wilkie, A.C., K.J. Riedesel, and J.M. Owens (2000) Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. *Biomass Bioenergy* **19**: 63–102.

Win, S.S., A. Impoolsup, and A. Noomhorm (1996) Growth kinetics of *Saccharomyces cerevisiae* in batch and fed-batch cultivation using sugarcane molasses and glucose syrup from cassava starch. *J. Ind. Microbiol*. **16**: 117–123.

Yokoi, H., T. Aratake, S. Nishio, J. Hirose, et al. (1998) Chitosan production from *shochu* distillery wastewater by funguses. *J. Ferment. Bioeng*. **85**: 246–249.

Yurekli, F., O. Yesilada, M. Yurekli, and S.F. Topcuoglu (1999) Plant growth hormone production from olive oil mill and alcohol factory wastewaters by white rot fungi. *World J. Microbiol. Biotechnol*. **15**: 503–505.

Zakowska, Z., and B. Gabara (1991) Changes in development and ultrastructure of *Aspergillus niger* mycelium, strain with increased tolerance to toxic substances from beet molasses. *Acta Microbiol. Pol*. **40**: 255–264.

4

FUNGAL METABOLISM OF PETROLEUM HYDROCARBONS

4.1 INTRODUCTION

There has been growing public concern regarding the release of petroleum hydrocarbons into the environment. Petroleum hydrocarbons are known to enter the environment frequently and in large volumes via several routes. One of the major and natural routes is the seepage from natural deposits that leads to the appearance of petroleum oil in marine environments. Another route includes production, storage, and transportation, which involves a significant potential for the accidental release of petroleum hydrocarbons. During the last decade, numerous incidents have been documented of petroleum leakage from underground storage tanks and pipelines. Marine spills also are a major source of water and coastal contamination.

Oil spills have become a global problem. Oil spills over 37800 liters (34000 kg) are reported in the International Oil Spill Database, which lists approximately $1.14 \times 10^6 \, m^3$ $(1.02 \times 10^9 \, kg)$ of oil spilled into U.S. marine waters since the early 1960s. This occurred in 826 incidents involving tankers, barges, and other vessels (Etkin, 1998) and about $0.76 \times 10^6 \, m^3$ $(0.68 \times 10^9 \, kg)$ of oil spilled onto U.S. soil from land pipelines (Etkin, 1997b). Estimated oil spills of $1.9 \times 10^6 \, m^3$ $(1.7 \times 10^9 \, kg)$ and over $0.76 \times 10^6 \, m^3$ $(0.68 \times 10^9 \, kg)$ from tankers have been reported in Europe (Etkin, 1997c) and Asia since 1965 (Etkin, 1997a). Oil spills over 37000 liters (34000 kg) into U.S. marine waters

from 1960 to 1997, including the number of spills and the amount spilled annually have been tabulated by Wang et al. (1999a).

Products derived from petroleum constitute the major source of energy for industry in and daily life (Harayama et al., 1999). Petroleum is also employed as a raw material in various products, such as cosmetics, paints, and plastics. Large quantities of petroleum oils and petroleum products enter marine and terrestrial environments, causing serious long-term concern regarding all forms of life and natural resources. Hundreds of papers have been published on the sources and impacts of spilled oils and petroleum products. Biodegradation of petroleum hydrocarbons in freshwater, terrestrial, and marine environments has been an area of intense interest and the subject of reviews by several authors (Prince, 1993; Atlas, 1995; Head and Swannell, 1999; Reddy and Quinn, 2001).

4.2   FATE OF OIL IN THE ENVIRONMENT

The quality and quantity of hydrocarbon mixtures and the properties of the affected ecosystem determine the persistence of petroleum hydrocarbons. In one environment hydrocarbons may persist for months, whereas under different environmental conditions the same hydrocarbons can degrade within a few hours or days. Low rates of biodegradation occur in nature and are limited by environmental factors (Bartha and Atlas, 1987). The daily rates of natural hydrocarbon degradation in adapted communities and pristine marine water are 0.5 to $50\,g/m^3$ and less than $0.03\,g/m^3$, respectively.

Bioremediation of petroleum hydrocarbon contaminants in marine habitats is usually limited by abiotic factors such as molecular oxygen and concentrations of nitrogen and phosphate. In anaerobic sediments, the rates of petroleum biodegradation are negligible, due to a lack of molecular oxygen, which is generally required for most microorganisms as an initial step in hydrocarbon metabolism. Marine habitats are not deficient in well-aerated oxygen.

The fate and behavior of spilled oil in natural environments are influenced by a number of physicochemical and biological factors, including evaporation, dissolution, photooxidation, microbial degradation, and interaction between oil and sediments. Physicochemical factors, such as volatilization, photolysis, sorption, dispersion, and mechanical emulsification, play an important role in petroleum biodegradation. The *combination* of these processes, known as *weathering*, reduces the concentration of hydrocarbons and causes changes in the chemical and physical properties of spilled oils. Alterations in the chemical composition of spilled oils has led to profound changes in the toxicity of oil and the biological impact of oil over time. The issue of biogenic and pyrogenic hydrocarbons from petrogenic hydrocarbons has been explored (Wang et al., 1999a).

4.3 COMPOSITION OF PETROLEUM HYDROCARBONS

Petroleum hydrocarbons contain a complex mixture of compounds that can be categorized into four fractions: saturates, aromatics, asphaltenes, and resins. The saturated fraction consists of straight-chain alkanes (normal alkanes), branched alkanes (isoalkanes), and cycloalkanes (naphthenes). The aromatic fraction includes volatile monoaromatic hydrocarbons such as benzene, toluene, and xylenes; polyaromatic hydrocarbons; naphthenoaromatics; and aromatic sulfur compounds, such as thiophenes and dibenzothiophenes. The asphaltene (phenols, fatty acids, ketones, esters, and porphyrins) and resin (pyridines, quinolines, carbazoles, sulfoxides, and amides) fractions consist of polar molecules containing N, S, and O_2. Asphaltenes are large molecules dispersed in oil in a colloidal manner, whereas resins are amorphous solids truly dissolved in oil. The relative distribution of these fractions depends on many factors, such as the source, age, geological history, migration, and alteration of crude oil. Hydrocarbons sensitive to microbial attack rank in the following order of decreased susceptibility: *n*-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes. The saturate fraction shows the highest rates of biodegradation, followed by light aromatics, with high-molecular-weight aromatics and polar compounds exhibiting the lowest rates of degradation. However, this pattern is not universal. A greater degradation loss of naphthalene than hexadecane in water–sediment mixtures from a freshwater lake (Cooney et al., 1985) and extensive biodegradation of alkylaromatics prior to detectable changes in the *n*-alkane profile in marine sediments (Jones et al., 1983) have been observed. Marine microbes also attack aromatic hydrocarbons during the degradation of crude oil. The variability in composition among crude oils and refined products contributes to the overall rate of biodegradation of both oil and its component fractions. Leahy and Colwell (1990) have reported the results of some of the examples.

Due to the presence of several compounds of complex structures, an increase in resistance of the residual mixture to further biodegradation occurs with progression of the biodegradation. Complete degradation of crude oils never occurs; a complex residue always remains. This residue, which is the color of black tar, contains a high proportion of asphaltic constituents. The residual mixture usually exhibits very low toxicity and bioavailability as long as it does not suffocate an area (Atlas, 1995).

4.4 METHODS OF ANALYSIS OF PETROLEUM HYDROCARBONS

A wide variety of instrumental and noninstrumental techniques have been established for the analysis of petroleum hydrocarbons. These include gas chromatography (GC), gas chromatography–mass spectrometry (GC-MS),

high-performance liquid chromatography (HPLC), size-exclusion HPLC, supercritical fluid chromatography (SFC), thin-layer chromatography (TLC), infrared spectroscopy (IR), isotope radio mass spectrometry, and gravimetric techniques. Among these, the GC techniques are the most widely used. One of the most enhanced techniques is capillary GC-MS, which can be used to analyze oil-specific biomarker compounds and polycyclic hydrocarbons. Currently, advances in computer technology have improved and optimized the accuracy and precision of data-handling capability, analytical data, and quality assurance/quality control.

Analytical techniques for the characterization of heavy oil contamination from soil matrices, including applications and limitations together with 90 references, are discussed by Whittaker et al. (1995). Sauer and Boehm (1995) presented a technical guide to chemical methods, including a tiered analytical approach, applications, and limitations together with over 110 references. Rapid HPLC methods (Krahn et al., 1993) and analytical techniques for fractionation and determination of hydrocarbon classes and groups, together with 109 references (Lundanes and Greibrokk, 1994), have been reviewed. The use of forensic environmental geochemistry (FEG) techniques in distinguishing various hydrocarbon fuels and solvents in samples has been described (Kaplan et al., 1997). Wang et al. (1999a) consolidated recent developments and advances in chemical and biomarker fingerprinting and data interpretation techniques, including 201 references. This includes the relative distribution pattern of petroleum hydrocarbons, analysis of "source-specific marker" compounds, diagnostic ratios of biomarkers, carbon isotope ratio measurements, principal component analysis and multivariate statistical analyses, and emerging techniques related to petroporphyrins and enantiomer analyses. Evolution of petroleum chemical fingerprinting techniques and new approaches to the characterization and differentiation among different petroleum sources have been discussed (Boehm et al., 1997). Wang and Fingas (1997) provided a brief survey and comparison of chemical fingerprinting techniques for the characterization of petroleum hydrocarbons, identification of oil spills, and assessment of environmental impacts. The total organic carbon (TOC) is used to screen soil samples for oil contamination. Morselli et al. (1999) presented a method of supercritical fluid extraction (SFE) for the determination of petroleum hydrocarbons in soil. The Site Characterization and Analysis Cone Penetrometer System (SCAPS) sensor technology is used for indirect detection of dense non-aqueous-phase liquid (DNAPL) plumes at the site (Kram, 1998). Tracy (1997) discussed immunoassay technology to screen oil contamination. Several field kits, such as the immunoassay-based EnviroGard, turbiditimetric-based PetroFlag test, and DR/2000 field kits have been evaluated as ways to measure total petroleum hydrocarbons (TPHs) and have been found to have certain limitations (Lambert et al., 2001).

4.5   ALTERNATIVE TREATMENT TECHNOLOGIES

Bioremediation is a very effective and promising technology for the treatment of petroleum hydrocarbon contamination. However, bioremediation is site-specific, and feasibility studies are required before the implementation of full-scale remediation. Bioremediation is a slow process and has many advantages, but it presents a challenge due to the heterogeneity of the contaminants, the presence of extreme concentrations of hydrocarbons, and variable site environmental conditions. Cleanup of petroleum-contaminated soils and aquifers is a priority task, due to the threat posed to soil fertility, drinking water, and groundwater (ASTM, 1995). A wide variety of physical, chemical, and biological technologies have been established for the decontamination of petroleum-contaminated sites (National Research Council, 1994; Alleman and Leeson, 1999). Of these technologies, engineered in situ bioremediation has received wide acclaim because it requires little energy, preserves the soil structure, and can detoxify the contaminants. Complete mineralization of hydrocarbons to innocuous end products is usually preferred compared to partial degradation to metabolites whose fate and effects are not known. In addition, the transfer of contaminants from one phase to another is not desirable for any successful remediation strategy. Engineered in situ bioremediation usually requires the stimulation of indigenous microflora by oxidants and nutrients. Petroleum can be degraded under aerobic and/or anaerobic conditions, but rapid degradation occurs under aerobic conditions.

During the last two decades, different soil and groundwater technologies related to the cleanup of petroleum hydrocarbons have been developed. Table 4.1 lists various treatment technologies for the removal or degradation of petroleum hydrocarbons in soil and groundwater. Each treatment technology has certain limitations and advantages at a given site. Norris et al. (1994) discussed design concepts for various types of bioremediation. The U.S. Environmental Protection Agency (EPA) has also published several monographs on different technologies, which can be useful in the cleanup of petroleum-contaminated sites. Modeling these technologies to achieve the removal efficiencies of contaminants is also described. Conversions of heavy crude oils can be induced, due to the action of microorganisms over periods of time or by the action of introduced microorganisms. Natural attenuation had become a popular and preferred cost-effective remedy for the degradation of hydrocarbons by the end of the last decade. Another cost-effective remedy is phytoremediation, which has also received wide public acclaim (McCutcheon and Schnoor, 2003). Pezeshki et al. (2000) provided an overview of the role of marsh macrophytes in the cleanup of petroleum hydrocarbons.

4.6   HYDROCARBON-UTILIZING YEASTS AND FUNGI

Hydrocarbons in the environment are degraded primarily by bacteria and fungi. The extent of hydrocarbon biodegradation by bacteria, yeasts, and

TABLE 4.1 Treatment Technologies for Removal or Degradation of Petroleum Hydrocarbons[a]

Treatment Technology	Soil	Groundwater	In Situ	Ex Situ
Air sparging		+	+	
Air stripping		+		+
Air biosparging	+	+		+
Carbon adsorption		+		+
Soil vapor extraction	+		+	
Soil flushing	+			+
Soil washing	+			+
Bioventing	+		+	
Incineration	+			
Natural attenuation		+	+	
Bioremediation	+	+	+	+
Phytoremediation	+	+	+	
Mycorrhizas	+			+
White-rot fungi	+			+

[a] A + denotes a treatment technology applicable to the matrix or location; a blank denotes a treatment technology not applicable to the matrix or location.

fungi is a function of the ecosystem and local environmental conditions. Bacteria and yeasts are generally predominant hydrocarbon degraders in the aquatic ecosystems, while fungi and bacteria are the main degraders in soil environments. However, yeasts and fungi have a significant effect on oil degradation, which occurs only under conditions that select against bacterial growth. A few studies are known that compare the level of hydrocarbon decomposition by bacteria and fungi in marine environments. Walker and Colwell (1974) reported the utilization of model petroleum at 0 and 5°C by bacteria, at 10°C by yeasts, and none by filamentous fungi. A hydrocarbon-utilizing fungus, *Cladosporium resinae*, degrades 20 to 40% of petroleum when added to the inoculum. The yeast populations increase in oil-contaminated estuarine sediments over a 4-month period but decline in open ocean waters in the presence of oil (Ahearn and Meyers, 1972). Scientists now accept that no single species of microorganisms will completely degrade any particular oil but this involves a consortium of microorganisms.

Hydrocarbon-utilizing yeasts and fungi are widely distributed in marine, freshwater, and soil habitats. Fungi constitute a minority in the marine environments, with increasing counts in nearshore regions, the intertidal zone, and salt marshes and mangrove areas. Ahearn and Meyers (1972) reported higher occurrences of yeasts and molds in rivers and lakes than in the ocean. Twenty-seven hydrocarbon-utilizing fungi from the marine environment (Floodgate, 1984) and 31 genera from soils (Bossert and Bartha, 1984) have been compiled. Common marine isolates are species of *Aureobasidium*, *Candida*, *Rhodotorula*, *Mortierella*, *Trichoderma*, and others. Kirk and

Gordon (1988) added the marine beach-adapted genera *Corollospora*, *Dendryphiella*, *Lulworthia*, and *Varicosporina*. Recently, fungal cultures inoculated on ceramic support media have shown an ability to degrade vapor-phase volatile organic compounds (VOCs) as their sole carbon and energy sources (Qi et al., 2002).

No authentic work is known on the existence of fungi on the surface of the ocean for a long time or at a 1-cm depth for petroleum degradation in nature. It is likely that degradation rates are different in arctic and temperate marine samples of fungi. The number of hydrocarbon-utilizing fungi is generally lower in winter than in summer. Fragmentary studies have shown a rise in population of fungi after oil spills. This indicates that certain fungi develop an enzymatic system after long contact with hydrocarbons. Pinholt et al. (1979) studied the microbial changes in soil during oil decomposition. They found an increase from 60% to 82% in oil-utilizing fungi. The minor role of fungi in a marine ecosystem can be attributed to lack of sporulation in marine habitats and morphological features for competitiveness in aquatic milieu and inhibition of spore germination by salinity. On the contrary, Kohlmeyer and Kohlmeyer (1979) reported the completion of the life cycle of several hundred species of obligate and facultative marine fungi in saline habitats and produced spore ornamentation that assist dispersal and colonization in seawater.

The ubiquitous distribution of *Cladosporium resinae* is known in oil-contaminated environments (Ahearn and Meyers, 1972). This distribution of yeasts and fungi suggests their important role in the degradation of oil spills in the environments. The application of waste oil to soil promotes the growth of *Graphium* and *Paecilomyces* spp. (Llanos and Kjoller, 1976). A group of terrestrial fungi (i.e., strains of *Acremonium*, *Graphium*, *Fusarium*, *Penicillium*, *Paecilomyces*, *Trichoderma*, and members of Sphaeropsidales) are capable of degrading crude oil hydrocarbons. Application of oil to soil produces changes in types of fungi (Westlake et al., 1978). Davies and Westlake (1979) found no relationship between the ability of yeasts and fungi to grow on pure *n*-alkane or naturally occurring whole crude oils containing these compounds. Oil-utilizing strains of *Aspergillus versicolor*, *Cephalosporium acremonium*, *Cunninghamella elegans*, and a *Penicillium* sp. from estuarine mud have been isolated (Cerniglia and Perry, 1973). About 96% (weight basis) of paraffin oil was utilized by strains of *C. elegans* during a 5-day period. *Cladosporium resinae* grows rapidly on *n*-alkanes and jet fuels (Cofone et al., 1973). Ahearn and Meyers (1976) established the role of yeasts and fungi in the removal of hydrocarbon from oil-contaminated environments.

4.7   FUNGAL METHODS OF ASSESSMENT

Successful biodegradation is a function of the presence of the activity of a fungal population capable of degrading target contaminants. Prior exposure

of contamination to an ecosystem has been correlated with hydrocarbon degraders. Unlike bacterial methods, less is known regarding fungal methods for bioremediation assessment. These methods are summarized below.

4.7.1   Fungal Enumeration

Preliminary analyses of the total heterotrophic and specific hydrocarbon-degrading fungal counts can provide a good understanding of soil biological conditions and the extent of acclimatization of indigenous fungal populations to site conditions. These indicate the viability of indigenous fungal population capable of supporting bioremediation. In addition to the preliminary analyses, monitoring fungal populations during soil remediation is important to discern fungal activity in hydrocarbon degradation. The literature to enumerate petroleum-degrading yeasts and fungi is inadequate and confusing. Sturman et al. (1995) discussed microbial enumeration methods, including heterotrophic plate counts, epifluorescence direct counts, hydrocarbon-degrader plate counts, most probable number (MPN) techniques, DNA (deoxyribonucleic acid) probes, lipid assays, metabolic indicators, and radioisotopic methods. Many of these methods are known for the enumeration of petroleum-degrading yeasts and fungi. *Candida albicans* OD-2 has been measured by direct counts (Fuentes et al., 1998). [^{3}H]Thymidine uptake, microautoradiography, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT)-reduction, adenosine triphosphate (ATP), and the frequency of dividing cells are employed to assess yeast activity.

Acidified wort agar is employed for the enumeration of yeasts introduced in the soil (Kulichevskaya et al., 1995). Fungi are also isolated by placing small pieces of tar balls on the surface of potato dextrose agar, V-8 agar, and Gorodkowa's media (Snellman et al., 1988). A basal salts medium is used for the growth of fungi on fuel oils. *Cladosporium resinae* is grown in a vortex-mixed 1.5-L fermentor in a mineral salts solution (Lindley and Heydeman, 1986). Fedorak et al. (1984) explored yeasts and fungi using Sabouraud's agar prepared in artificial seawater and provided a distribution of yeast and fungal colony-forming units (CFU) in marine samples. Artificial seawater agar slants, each with different carbon and petroleum sources, were prepared to study patterns of substrate utilization (Kirk and Gordon, 1988). Malt-agar chloramphenicol medium for the growth of fungal populations and media specific for hydrocarbon-adapted fungi (HAF) have also been employed (Oudot et al., 1993). Malt is the source of carbon in the first medium, and hydrocarbons for HAF medium. A slant culture method has been used for the assimilation of *n*-hexadecane by yeast (Middelhoven et al., 2000). Middelhoven et al. (1992) employed yeast enrichment methods for benzene degradation in acidic soils. A mixture of kerosene and automotive gas oil was evaluated for routine enrichment for the isolation of a yeast strain (Okpokwasili and Amanchukwu, 1988).

A yeast medium was considered for the isolation of heterotrophic yeasts and fungi by Walker and Colwell (1974, 1976), who also enumerated the

counts of petroleum-degrading yeasts and fungi and the efficiency of various media (SGO, MPN, yeast agar, and OA No. 2) from estuarine and marine samples. Washing the inoculum did not improve the recovery of petroleum degraders. Marine arenicolous fungi were also enumerated (Kirk, 1983).

4.7.2 Respirometric Tests

Mineralization studies involving the measurement of total CO_2 production is a useful source of knowledge on the potential of hydrocarbon degradation in contaminated soils. This approach provides a rapid test for the determination of various biological treatment options, such as the effect of nutrient addition and fungal inoculation. This test is also used to confirm active hydrocarbon degradation during full-scale bioremediation. Simple respirometric flask methods or expensive automated equipment for several samples simultaneously can be employed for the monitoring of O_2 consumption and/or CO_2 evolution rates. The Biometer flask test can be conducted to assess mineralization rates from a small amount of soil (Balba et al., 1998).

A radiorespiratory method using n-[1-^{14}C]hexadecane and [9-^{14}C]phenanthrene verified the mineralization of aliphatic compounds by filamentous fungi (April et al., 2000). A method was given for the recovery of ^{14}C in aqueous, organic, and cell fractions following incubation of fungi in a liquid culture with a crude oil spiked with n-[1-^{14}C]hexadecane or [9-^{14}C]phenanthrene. Bottles closed with silicon stoppers were used to determine the rate of respiration, and the evolution of CO_2 was measured after an hour of incubation (Kulichevskaya et al., 1995). Radiorespirometric studies can be performed to produce [14]CO_2 from ^{14}C-labeled hydrocarbons by nine fungal and three yeast isolates in a flask (Fedorak et al., 1984). Mineralization of hexadecane was also studied by radiorespirometry using fungal strains (Kirk and Gordon, 1988).

4.7.3 Soil Microcosm Tests

Soil microcosm tests can be conducted in simple jars of contaminated soil from highly sophisticated systems. Good results are expected when real environmental conditions are incorporated in the model of microcosm design. These tests include sterile treatments as appropriate controls to separate the effects of abiotic hydrocarbon loss from actual biodegradation. Such tests are employed to assess the biodegradation potential and development of models for predicting the fate of hydrocarbons. Mathematical equations can be formulated to describe the kinetics of each process involving the transformation of constituents of specific hydrocarbons. The rate of degradation and metabolic products can be monitored.

A soil microcosm test using fungi has been designed by Yateem et al. (1998). A spreading technique was employed to enumerate the total viable aerobic microorganisms in the soil extract and its serial dilutions. The experiments were conducted in eight 4-L sterilized glass flasks loaded under a

laminar flow bench with 1 kg of contaminated soil (Colombo et al., 1996). Three treatments were tested for the influence of temperature on abiotic and biotic hydrocarbon loss in soil using the yeast *Yarrowia lipolytica* (Margesin and Schinner, 1997). The water content was adjusted with sterile water to 60% of a soil's maximum water-holding capacity. Competition experiments conducted with *n*-alkane utilizers, yeasts, and bacteria confirmed the overgrowth of yeasts over bacteria in sandy soil (Schmitz et al., 2000). Acidification favors the colonization of yeasts. It can be counteracted by the addition of bentonite. Different strains of yeasts reveal different levels of competitiveness. Strains of *Arxula adeninivorans*, *Candida maltosa*, and *Yarrowia lipolytica* showed more overgrowth than did strains of *Candida tropicalis*, *Candida shehatae*, or *Pichia stipitis*. Two strains of *C. maltosa* and *Y. lipolytica* coexisted during several serial transfers under microcosm conditions.

Candida lipolytica has been introduced in oil-contaminated soils for population dynamics (Kulichevskaya et al., 1995). Soil is moistened to 60% of its total moisture capacity mixed with crude oil (0.1 cm^3/g soil) and mineral salts. Soil is inoculated with lyophilized biomass to a concentration of 3 mg/g soil, which corresponds to a population density of 10^8 cells/g soil. Soils are placed in different bottles and incubated at 22°C for 40 days with humidity maintained at a constant level.

4.7.4   Miscellaneous Tests

Other tests (i.e., dehydrogenase activity and biomarker compounds) are used for microbial assessment (Balba et al., 1998). The assay of dehydrogenase in contaminated soil indicates the possible inhibitory effect of contaminants on a soil's microbial activities. The ratios of hydrocarbon compounds within a complex hydrocarbon mixture can be employed to assess hydrocarbon degradation. Unfortunately, no studies are available on these tests by petroleum-utilizing yeasts and fungi.

4.8   HYDROCARBON METABOLISM BY YEASTS AND FUNGI

Bacteria and fungi exhibit different abilities to metabolize hydrocarbons. Both yeasts and bacteria show decreasing abilities to metabolize alkanes with increasing chain length. Filamentous fungi do not exhibit preferential degradation for a particular chain length. Similar patterns of hydrocarbon metabolism are known in bacteria and fungi, but there is considerable variability among individual isolates. Hyphal structures and increases in surface area allow better penetration of the hydrocarbons and hydrocarbon-impregnated soil aggregates that may be anoxic internally. Many such species of fungi are xero- and osmotolerant. Certain fungi contain extracellular enzymes that may assist in the initial degradation of hydrocarbons. Yeast and fungal degradation of petroleum hydrocarbons are noted in Table 4.2.

TABLE 4.2 Yeast and Fungal Degradation of Petroleum Hydrocarbons

Fungus/Yeast	Petroleum Hydrocarbon	Medium/Soil	Assimilation/ Degradation Rate (%)	Duration (days)	Reference
Cladophialophora sp. strain T1	BTEX mixture	Mineral salts medium	All degraded except benzene	30	Prenafeta-Boldu et al., 2002
Trichosporon veenhuisii sp. nov.	Hexadecane	Slant culture method	Assimilated	14	Middelhoven et al., 2000
Aspergillus fumigatus (four strains)	n-[1-^{14}C]Hexadecane	Liquid medium	21–43 as $^{14}CO_2$	14	April et al., 2000
Neosartorya fischeri (two strains)			5–13 as $^{14}CO_2$		
Oidiodendron griseum (three strains)			5–32 as $^{14}CO_2$		
Philophora americana			37 as $^{14}CO_2$		
Pseudallescheria boydii			29 as $^{14}CO_2$		
Candida lipolytica	Oil	Contaminated soil	3–10µg CO_2-C/h per gram of soil	5–7	Kulichevskaya et al., 1995
Candida tropicalis PFS-95	Crude oil		69	16	Ijah, 1998
Phanerochaete chrysosporium	TPHs	Soil microcosm test	78	365	Yateem et al., 1998
Pleurotus ostreatus			53		
Coriolus versicolor			69		
Aspergillus terreus	Aliphatic and aromatic	Natural soil contaminated with 10% crude oil	86 and 100	90	Colombo et al., 1996
Fusarium solani			74 and 100		
Arxula adeninivorans	Benzene compounds	Slant culture medium			Middelhoven et al., 1991, 1992
Yeasts	Benzene compounds	Natural soil			
Eupenicillium javanicum	TPHs	HAF medium	30	30	Oudot et al., 1993
Graphium putredinis			33		
Aspergillus flavipes			26		
Candida sp.	Kerosene and AGO	Mineral salts medium	Degraded	14	Okpokwasili and Amanchukwu, 1988
Graphium sp., *Tetracoccosporium*	Fuel oils no. 2 and no. 4	Basal salts medium	Assimilated	15	Snellman et al., 1988

A soil fungus, *Cladophialophora* sp. strain T1, does not metabolize benzene, but alkylated benzenes such as toluene, ethylbenzene, and xylenes are degraded by a combination of assimilation and cometabolism (Prenafeta-Boldu et al., 2002). Degradation of *para*-xylene does not occur in complex BTEX mixtures, but 60% depletes when combined with toluene. ^{1}H-NMR metabolic profiles revealed the formation of phthalates as end products of *ortho*- and *meta*-xylenes. Substrate interactions of toluene, ethylbenzene, and xylenes indicate their degradation at the alkyl side chain by the monooxygenase enzyme. Fungi are well known to grow on the water-soluble fraction of petroleum fuels, but only aliphatic hydrocarbons support fungal growth. This appears to be the first report of a fungus growing on aromatic hydrocarbons from a gasoline water-soluble fraction containing all six BTEX compounds. During the metabolism of toluene by *Cladophialophora* sp. strain T1, hydroxylation of toluene to benzyl alcohol and subsequent transformation to benzoic acid occurred prior to hydroxylation and cleavage of the aromatic ring (Prenafeta-Boldu et al., 2001).

Trichosporon veenhuisii sp. nov., an alkane-assimilating anamorphic basidiomycetous yeast, has the ability to assimilate several aliphatic and aromatic compounds as a sole source of carbon and energy (Middelhoven et al., 2000). Introduction of *Candida lipolytica* into oil-contaminated soil can increase the rate of oil degradation (Kulichevskaya et al., 1995). An increase in rates of respiratory activity of yeasts associated with growth on oil-containing hydrocarbons in all three oil-polluted soils has been observed. Gray forest soils provide the most favorable conditions for the development of introduced yeasts. In soddy-podzolic soil, active proliferation and oil degradation are retarded as a consequences of inadequate aeration by inadequate structuring and heavy mechanical composition of this soil. This demonstrates the substantial intensification of oil degradation in soil by the hydrocarbon-oxidized yeasts introduced. An ability to degrade hydrocarbons was noted in species of six orders of Ascomycetes (April et al., 2000). Species attacked compounds within the aliphatic fraction of crude oil, ranging from C12 to C26. All species degraded the aliphatic fraction of petroleum hydrocarbons. This was the first report in mineralizing hydrocarbons by *Neosartorya fischeri*, *Oidiodendron griseum*, and *Phialophora americana*.

Three white-rot fungi (i.e., *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, and *Coriolus versicolor*) were shown to have the ability to degrade oil in contaminated soil (Yateem et al., 1998). Total petroleum hydrocarbons (TPHs) were reduced considerably by all three fungal cultures after 4 months under nitrogen-rich conditions. *P. chrysosporium* degraded all the BTEX components either individually or in a composite mixture (Yadav and Reddy, 1993). Degradation occurred under nonligninolytic conditions in malt extract medium in which extracellular lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) are not produced. In vitro studies by wild or mutant types also confirmed that LiP and MnP are not involved in BTEX degradation. Uniform ring-labeled [^{14}C]benzene and [^{14}C]toluene underwent

greater mineralization to $^{14}CO_2$. Compared to natural soil microflora, pure fungal cultures reduced hydrocarbon residues by 26 to 35% in 90 days (Colombo et al., 1996). In general, Imperfect fungi isolated from contaminated soils show somewhat higher efficiency. Three unadapted and indigenous species of ligninolytic fungi, such as *Coriolopsis rigida*, *P. ostreatus*, and *Trametes villosus*, effectively and comparably degraded the aliphatic and aromatic components. Multivariate analysis of 22 parameters indicated a reactivity trend in the components during oil degradation as follows: low-molecular-weight *n*-alkanes > phenanthrene > 3,2-methylphenanthrenes > intermediate-chain-length *n*-alkanes > longer-chain-length *n*-alkanes > iso-prenoids ≈ 9,1-methylphenanthrenes. Irrespective of degrading capacity, all species of fungi exhibit this decomposition sequence.

Of seven strains of filamentous fungi, the H1 and H7 strains of *Hormoconis resinae* and the H3 and H4 strains of *Penicillium* were shown to grow on derivatives of petroleum (Laborda et al., 1996). The molds degraded the *n*-alkane components of fuel and H7 also degraded the branched hydrocarbons, pristane and phytane. Incubation with heating oil resulted in the detection of a large number of microbodies in the cytoplasm of H1 and H7, revealed by transmission electron microscopy. Several benzene compounds were assimilated by Imperfect ascomycete, *Arxula adeninivorans* (Middelhoven et al., 1991). Of several hydrocarbon-assimilating fungi, the most active strains in the assimilation of saturates and aromatics were shown to be *Beauveria alba* and *Penicillium simplicissimum* (Chaineau et al., 1999). Hydrocarbons were also assimilated by a species of *Fusarium* (Nair and Lokabharathi, 1977).

Several strains of hydrocarbon-assimilating fungi were isolated from tropical environments, a forest soil, and the sediments of a river contaminated by petroleum spills in a study by Oudot et al. (1993). The most active strains exhibiting biodegradation of total petroleum exceeding 25% were *Eupenicillium javanicum*, *Graphium putredinis*, and *Aspergillus flavipes*. These strains provided the most efficiency in assimilation of saturates exceeding 40% and aromatics exceeding 30%. *Emericella nidulans*, *Eupenicillium javanicum*, *Gliocladium virens*, and *Aspergillus fumigatus* degraded the resins significantly (15 to 28%). *Emericella nidulans, Eupenicillium javanicum*, *Graphium putredinis*, and *Acremonium* spp. degraded about 15 to 40% of asphaltenes. However, the petroleum resins and asphaltenes are considered as resistant or refractory to biodegradation. The mixed culture of the soil strains is as active as the most efficient mono-specific cultures, whereas the mixed culture of the river strains is not very active. The activity is closer to *Eupenicillium* than to the best-performing strain of *E. nidulans*. This implies the nonpredominance of the most efficient strain during competition of growth.

A degradative loss of some components of kerosene hydrocarbon by an isolate of *Candida* species has been proved (Okpokwasili and Amanchukwu, 1988). Weathering showed a greater effect on kerosene than on automotive gas oil (AGO). The best carbon source for growth was AGO, followed by kerosene, alcohol, and acetaldehyde. This indicates the capability of growth

of a soil yeast isolate on alcohols and aldehydes produced as a result of oxidation of the hydrocarbons. This may be a potential candidate strain for seeding in the biodegradation of oil-polluted terrestrial ecosystems.

Several filamentous fungi were isolated from half of 300 tar balls collected from the North Atlantic and North Pacific Oceans (Snellman et al., 1988). *Tetracoccosporium* produced the greatest mycelia biomass. Both *Lophotrichus* and *Tetracoccosporium* assimilated the components of fuel oil no. 4, irrespective of oil concentration. The ability of 54 strains in 30 species and 23 genera of filamentous marine Ascomycetes and Deuteromycetes, representing arenicolous, caulicolous, and lignicolous fungi, to assimilate anthropogenic petroleum compounds has been examined (Kirk and Gordon, 1988). Of 18 arenicolous species, 14 showed growth on hexadecane as the sole carbon source. These species also mineralized the labeled hexadecane, releasing $^{14}CO_2$. None of nine caulicolous and only three of 27 lignicolous marine fungi showed growth on hexadecane as a carbon source. The 14 arenicolous and three lignicolous species that exhibited growth on hexadecane also assimilated 1-hexadecane, pristane, and to a lesser extent, tetradecane as sole carbon sources. *Zalerion maritimum* is the only species that utilized dodecane and decane, whereas *Varicosporina ramulosa* formed asexual fruiting bodies on these hydrocarbons. Most of the hexadecane-utilizing species revealed emulsification after 6 weeks, implying the penetration of the hydrocarbon droplets by the mycelia. Of 224 fungi and 74 yeasts isolated from marine water and sediment samples, only 63 fungi and 3 yeast isolates utilized the *n*-alkane profile of crude oil (Fedorak et al., 1984). About a 50% reduction in the number of isolates was detected when the temperature was reduced from 20°C to 8°C and the incubation time was doubled. Of these, 47 isolates belong to the genera of *Penicillium*. Other isolates that showed an ability to grow on this oil belong to the genera *Aspergillus*, *Beauveria*, *Paecilomyces*, and *Verticillium*. Yeast blooms on the surface of oil slicks due to the growth of indigenous yeasts (Ahearn and Meyers, 1976). However, the growth of common marine yeasts is slow compared to that of yeasts isolated from aquatic ecosystems contaminated with oil. Geofungi revealed faster growth than that of marine species in culture (Kohlmeyer and Kohlmeyer, 1979). However, little is known about the ecological significance of hydrocarbon-utilizing marine fungi, despite their richness, adaptiveness, and diversity of chemical activities.

Two fungal strains on crude oil as the sole source of carbon were exposed to three crude oils that showed a range of mutagenic activity by Rudd et al. (1996). Extracts of fungal incubation with the various crude oils were tested for mutagenic activity by spiral *Salmonella* assay. *Cunninghamella elegans* or *Penicillium zonatum* degraded Pennsylvania crude oil and its mutagenicity was reduced significantly. The moderately mutagenic West Texas Sour crude oil showed little change in mutagenicity during incubation with either of these fungi. The slightly mutagenic Swanson River Field crude oil from Cook Inlet, Alaska, became more mutagenic during incubation with *Cunninghamella*

elegans. Measurement of mycelial mats during growth on crude oils indicated about 25% biodegradation of crude oil.

4.9   TAXONOMIC RELATIONSHIP OF HYDROCARBON-UTILIZING YEASTS AND FUNGI

Yeasts and fungi are a diverse group of organisms for hydrocarbon utilization. Bartha and Atlas (1977) listed 14 genera of fungi isolated from an aquatic environment that had been shown to utilize hydrocarbons. These included species of *Candida*, *Rhodotorula*, and *Sporobolomyces*. Davies and Westlake (1979) studied the growth capability of 60 fungal isolates on *n*-tetradecane, toluene, naphthalene, and seven crude oils of various compositions. Forty cultures, including 28 soil isolates, exhibited growth on one or more of the crude oils. The common genera isolated from soils are those producing abundant small conidia (e.g., *Penicillium* and *Verticillium* spp.). Oil-degrading strains of *Beauveria bassiana*, *Mortierella* spp., *Phoma* spp., *Scolecobasidium obovatum*, and *Tolypocladium inflatum* were also isolated.

Certain yeasts strains (i.e., *Candida*, *Rhodotorula*, *Saccharomyces*, *Sporobolomyces*, and *Trichosporon*) capable of utilizing hydrocarbons have been isolated (Ahearn et al., 1971; Cook et al., 1973). *Cladosporium resinae* isolated from the soil (Cooney and Walker, 1973) was found to be a contaminant of jet fuels (Bailey and May, 1979). Komagata et al. (1964) studied 50% of yeasts for hydrocarbon-degrading ability and found 56 species that can utilize hydrocarbons, almost all belonging to the genus *Candida*.

The ability of yeasts and fungi to utilize hydrocarbons as the sole source of carbon and energy is well established in the literature. Fungi capable of utilizing hydrocarbons are limited to occur within the orders Mucorales (Zygomycetes) and Moniliales (Hyphomycetes). In general, isolates of genera *Aspergillus* and *Penicillum* are rich in assimilating hydrocarbons. These fungi also grow on *n*-paraffins with 10 carbon atoms rather than on petroleum fractions or aromatic compounds. More than 50% of the isolates belong to Hypocreaceae and Trichocomaceae in Ascomycetes (April et al., 2000). Both of these groups contain several species, which are osmo- as well as xerotolerant. The most xerotolerant species belong to the genus *Aspergillus* and several species of *Penicillium* and *Fusarium*. A high salt concentration can lead to low water availability by lowering the osmotic potential. Species of *Aspergillus* and *Penicillium* can also grow at a lower osmotic potential, −50 MPa (Dix and Webster, 1995). *Pseudallescheria boydii*, belonging to Microascaceae, is also osmotolerant, due to its growth at a high salt concentration of 2% NaCl and 5% $MgCl_2$ (de Hoog et al., 1994).

At present, the ability to utilize hydrocarbons is widespread among diverse fungal populations. Hydrocarbons are naturally occurring organic compounds, and it is not surprising that yeasts and fungi have evolved the ability to utilize these compounds. When natural ecosystems are contaminated with

petroleum hydrocarbons, the indigenous microflora is likely to contain fungal or microbial populations of differing taxonomic relationships that are capable of degrading the contaminating hydrocarbons.

4.10   FACTORS AFFECTING METABOLISM OF PETROLEUM HYDROCARBONS

The biodegradation of petroleum hydrocarbons in the environment is determined largely by abiotic factors. Factors affecting microbial degradation of petroleum hydrocarbons have been the subject of considerable interest during the past two decades. Fungi can withstand fairly wide fluctuations in environmental conditions. The various factors that influence the growth rates and enzymatic activities of yeasts and fungi also affect the rates of petroleum degradation. Various factors influencing the fungal degradation of petroleum hydrocarbons have been recognized. These factors are divided into three categories: (1) physicochemical (physical nature, solubility, size and concentration, oil–water interface, volatility, etc.), (2) environmental (temperature, pH, light, salinity, oxygen level, nutrients, soil/sediment type, etc.), and (3) fungal (distribution in an area, population density adaptation, uptake, genetic composition, microbial interactions, etc.). Important factors affecting the fungal metabolism of petroleum hydrocarbons are summarized next.

4.10.1   Physical Nature

The physical nature of hydrocarbons has a great effect on the process of biodegradation. The hydrocarbon-degrading yeasts and fungi act primarily at the oil–water interface. However, yeasts and fungi can be found growing over the entire surface of an oil droplet, and growth does not occur within oil droplets in the absence of entrained water. The movement of emulsion droplets through a water column allows the uptake of oxygen, nutrients, and oil to fungi.

4.10.2   Temperature

Based on temperature, hydrocarbon degradation can occur under three conditions: psychrophilic, mesophilic, and thermophilic. In general, most fungi are mesophilic in isolation, growth, and reproduction. Temperature is essential for the growth requirements of certain yeasts and fungi along with petroleum as a substrate. Low temperatures generally retard the rates of volatilization of low-molecular-weight hydrocarbons, some of which are toxic to yeasts and fungi. Fungi also show a propensity to withstand dry environments and high temperatures and thus appear to be suitable for the remediation of these contaminated areas. The effects of temperature are also

considered with other factors, such as the quality of the hydrocarbon mixture and the composition of the fungal population.

Temperature influences diesel oil biodegradation by the psychrotrophic yeast *Yarrowia lipolytica* in a mineral medium and in soil (Margesin and Schinner, 1997). Abiotic loss of diesel oil increases with incubation time and with temperature and is lower in a mineral medium than in soil. This amounts to a loss of 20 to 45% (5000 mg/kg soil dry weight) in soil and 15 to 27% in liquid media after 30 days at 4 to 30°C. Higher biodegradation activity is detected in liquid culture than in soil at all temperatures. *Y. lipolytica* was shown to degrade about 20% of the diesel oil in soil. The degradation activity was highest at 10 to 15°C after 5 days and at 4°C after 10 days. BTEX degradation by *Phanerochaete chrysosporium* was higher at 25°C than at 37°C (Yadav and Reddy, 1993).

4.10.3 pH

Several fungi grow well at pH levels of 4 to 5 and yeasts at 3 to 4 and are more tolerant of acidic conditions, where it is difficult for bacteria to thrive. *Cladosporium resinae* grows slowly in seawater and requires organic stimulation for growth (Neihof and May, 1983). In certain cases, BTEX degradation by *P. chrysosporium* is little affected by pH variations between 4.5 and 7.0 (Yadav and Reddy, 1993).

4.10.4 Oxygen

Fungi are both aerobic and anaerobic but grow well under aerobic conditions. Oxygen is necessary for the mineralization of hydrocarbons in estuarine sediments. The rates of hydrocarbon degradation are reduced with decreasing oxygen reduction potential. Hydrocarbons persist in reduced sediments for longer periods than in aerated surface layers. The initial steps in the catabolism of aliphatic, cyclic, and aromatic hydrocarbons by fungi involve oxidation of the substrate by oxygenases and molecular oxygen. Thus, aerobic conditions are necessary for the oxidation of hydrocarbons in the environment. Substantially greater degradation of all BTEX compounds occurs in static than in shaken liquid cultures (Yadav and Reddy, 1993). Negligible rates of biodegradation of hydrocarbons occur in anaerobic environments.

4.10.5 Nutrients, Dispersants, and Biosurfactants

Little is known about petroleum degradation in the presence of nutrients by yeasts and fungi. Low nitrogen levels, low pH, low moisture content, and inadequacy of certain nutrients favor the development of fungi. In oil slicks, a proportion of carbon is readily available for yeast growth within a limited area. Since nitrogen and phosphorus components are essential for incorporation into yeast or fungal biomass, the availability of these nutrients within the

hydrocarbon location is important. In many cases, the supply of nitrogen and phosphorus depends on the diffusion in the oil slick. The degradation of hydrocarbons can be accelerated by the addition of specific urea-phosphate, N-P-K fertilizers, and so on, for fungal growth. Fungal cells usually contain less nitrogen than bacterial cells and thus fungi can act favorably in ecosystems that have a low nitrogen content. Many fungal isolates grow equally well in laboratory diesel–water systems with or without an additive (Bento and Gaylarde, 2001). However, the composition of fungal cells can be represented empirically by $C_{10}H_{17}O_6N$.

Dispersants have demonstrated a positive effect on rates of degradation by dissolution and emulsification of hydrocarbons. Fungal levels in analytical freshwater ponds are enhanced significantly after the addition of oil–dispersant mixtures (Sherry, 1984). Some dispersants are toxic and inhibitory to yeasts and fungi. The use of natural biosurfactants produced by yeasts or fungi has a marked potential in such biospheres (Lindley, 1991, 1994). The chemical nature of biosurfactants produced by yeasts appears to be that of glycolipids. The physical properties of such compounds play an important role in petroleum degradation. The major action of these biosurfactants is to increase the available surface area of the hydrocarbon phase for uptake transport by fungi.

Yeast extract and malt extract enhance cell growth and overall *n*-alkane degradation by the polyethylene-degrading fungus *Penicillium simplicissimum* YP (Yamada-Onodera et al., 2002). Squalane is more favorable than pristane to long-chain *n*-alkane degradation when the cell density is higher. The degradation efficiency is enhanced further using Plysurf A210G as the dispersant and supplementing with a high concentration (0.3%) of malt extract. The fungus can also grow in the presence of pristane, squalane, and *n*-alkanes with a chain 20 to 50 carbons long. This fungus has a potential for application in bioremediation of contaminated areas containing recalcitrant long-chain alkanes.

4.11   FUNGAL MECHANISMS OF METABOLISM OF PETROLEUM HYDROCARBONS

Hydrocarbon-degrading fungi have been known for more than five decades, but our understanding of mechanisms involved in the catabolic breakdown of hydrocarbons is still incomplete. Certain aspects of fungal metabolism of hydrocarbons are similar to the metabolism of both higher eukaryotic organisms and bacteria (Smith and Rosazza, 1974). Like bacteria, fungi participate in the transformation reactions and assimilate hydrocarbons as the sole source of carbon and energy for growth, resulting in the formation of carbon dioxide. The fungal mechanisms of metabolism of petroleum hydrocarbon have been discussed by many researchers and divided into four categories.

4.11.1   Aliphatic Hydrocarbons

The most thoroughly studied aliphatic hydrocarbons are *n*-alkanes of C10 to C20 that can be metabolized rapidly. Methyl alkanes may also be metabolized but with less growth. The degradation of long-chain alkanes greater than C24 can occur in complex mixtures, but no major studies are known to support fungal growth. Kremer and Anke (1997) listed the alkane-assimilating genera of fungi.

As with most microorganisms, the most common major pathway of alkane metabolism is *monoterminal oxidation* to the corresponding alcohol, alde-hyde, and fatty acid. This involves a mixed function of alkane monoxygenase, nicotinamide adenine dinucleotide (NAD)-dependent alcohol, and aldehyde dehydrogenases. The initial mode of attack is at the site of the terminal methyl group by alkane monoxygenase involving the insertion of molecular oxygen and an electron transfer system. This array involves the combination of cyto-chrome P450 as the terminal oxidase and nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450-reductase as the electron transfer component. The following dehydrogenase steps to long-chain alcohols and aldehydes yield fatty acids. Fatty acids are also generated due to alkane assimilation to function as building blocks (Brakemeier et al., 1995). Further catabolism involves the initial activation of fatty acids to acyl-CoA ester via an acyl-CoA synthetase and β-oxidation and yields to acetyl-CoA. The acetyl-CoA can either act as an anabolic precursor or is further catabolized to CO_2 via a tricarboxylic acid cycle. The possibility exists of terminal oxidation to the corresponding fatty acids followed by β-oxidation for alkane mineraliza-tion in marine fungi (Singer and Finnerty, 1984). The slow alkane utilization by marine fungi indicates a lack of constitutive enzymes for the initial oxida-tion, and this requires further investigation.

Although a monoterminal pathway is most commonly encountered, certain species possess alternative pathways. Some yeasts and species of *Aspergillus*, *Botrytis*, and *Penicillium* can oxidize both terminal methyl groups of aliphatic hydrocarbons to produce long-chain dicarboxylic acid, which is known as *diterminal oxidation*. This oxidation may occur either simultaneously or the ω-methyl group is oxidized after formation of the fatty acids. Several species of *Candida* accumulate dioic acid on alkanes (Uchio and Shiio, 1972; Il'Chenko, 1984). This pathway has been exploited for the overproduction of dicarboxylic acid.

Another mechanism is *subterminal oxidation*, which is initiated in the formation of a secondary alcohol, followed by oxidation to the corresponding ketone and ester, which cleaves to yield both a primary alcohol and acetate. Subterminal oxidation is noted in species of *Aspergillus*, *Cladosporium*, *Peni-cillium*, *Verticillium*, *Cunninghamella echinulata*, *Fusarium lini*, *Mortierella isabellina*, and *Rhizopus nigricans*, (Hoffmann and Rehm, 1976a; Hoffmann et al., 1977; Yi and Rehm, 1982; Kremer and Anke, 1997). Subterminal

oxidation can occur in various positions, with formation of a mixture of secondary alcohols whose further metabolism yields the corresponding primary alcohols and organic acids. Further metabolism of alcohol employs steps of dehydrogenation and β-oxidation. A scheme of the various steps involved in alkane oxidation by Mucorales has also been proposed (Pelz and Rehm, 1973; Hoffmann and Rehm, 1976b).

4.11.2   Aromatic Hydrocarbons

The metabolism and transformation of aromatic hydrocarbons by fungi have been well established during the past two decades. Several species of fungi have shown the ability to assimilate a wide range of aromatic hydrocarbons. The oxidation of aromatic hydrocarbons is initiated with an epoxidation to arene oxides by cytochrome P450–dependent monoxygenases. Several metabolic pathways are recognized for the degradation of aromatic hydrocarbons (see Chapter 8).

4.11.3   Cooxidation of Hydrocarbons

In certain cases, fungi can initiate breaking or modifying complex hydrocarbons despite nonoccurrence of growth. Some degree of partial conversion may occur in the presence of an alternative substrate acting as a source of both carbon and energy. Few fungi can metabolize cycloalkanes. Certain studies did not show the toxicity of cycloalkanes to representative yeasts. The metabolism of cycloalkanes in fungi appears to proceed via cooxidation. Studies of fungi capable of growth on cyclic aliphatics are rare. Cyclohexane has no terminal methyl groups and is assimilated by a mechanism similar to subterminal oxidation (Perry, 1979). This mechanism involves the formation of dicarboxylic acid, which is subsequently metabolized by β-oxidation. The possibility of cooxidation of alkanes by lignicolous marine fungi is correlated, owing to much better growth on glucose with hydrocarbons (Kirk and Gordon, 1988).

4.11.4   Uptake of Hydrocarbons

The uptake of hydrocarbons involves penetration of insoluble substrate into a cell with a variety of mechanisms. One of the most acceptable mechanisms is via the transport process. Yeasts accumulate fatty acids within cell material, and filamentous fungi show intracellular aggregation of nonassimilated alkanes during conditions of excessive carbon. At the cell surface, penetration can occur through pores and channels, and hydrocarbon moves toward the plasmalemma. If the initial enzymes are present in the plasmalemma, the aldehydes or fatty acids may enter cytoplasm as a result of activation of hydrocarbons, but the cytochemical location of enzymes does not agree with such a mechanism.

Membrane-bound, electron-dense inclusion bodies (vesicles) in *Cladosporium resinae* have been detected during growth on alkanes (Cooney et al., 1980). Lindley and Heydeman (1986) described dodecane uptake by whole cells of *C. resinae* in two stages. Here, passive adsorption of the hydrocarbon to the outer cell surface follows by a mechanism that obeys Michaelis–Menten saturation kinetics. This two-stage uptake mechanism is similar to that of the yeast *Candida tropicalis* (Kappeli and Fiechter, 1981). This yeast produces a fatty acid complex that emulsifies the hydrocarbon and thus results an induced uptake of pure hydrocarbon. The alkane solubilization is indicated by an aqueous phase during transport by *Candida lipolytica* (Goma et al., 1973). Three steps of alkane metabolism are stated: adsorption to the cell surface, movement through the rigid cell wall via pores or channels, followed by alkane movement via pinocytosis to microbodies and other sites of oxidation (Singer and Finnerty, 1984). They also show the production of extracellular products in *C. lipolytica*, which induces both emulsification and uptake of hydrocarbons. The exact nature of biochemical mechanism of alkane metabolism remains unknown, but the available knowledge suggests the involvement of the physicochemical nature of the cell wall. In eukaryotic microorganisms, hydrocarbon catabolism is associated with distinct and induced ultrastructural characteristics (Fukui and Tanaka, 1979). The most important changes include the appearance of crystalline peroxisomes in yeasts and fungi. During alkane degradation by *Candida* and other species, peroxisomes are induced in large proportions of the cell volume and in high numbers are similar to methylotrophic yeasts and unlike *Saccharomyces cerevisiae*. The intracellular distribution of various biochemical pathways is located in diverse microbodies (peroxisomes, microsomes, and mitochondria) associated with alkane metabolism in *C. tropicalis* (Tanaka et al., 1982).

4.12   OXIDATION OF PETROLEUM HYDROCARBONS BY FUNGAL ENZYMES

Little is known of the enzymatic oxidation of petroleum hydrocarbons, and this topic is emerging due to regio- and stereoselectivity and mild physiological conditions. Enzymatic oxidation can take the upper hand when success is not achieved with chemical catalysts. Faber (1997) discussed biocatalytic oxidation reactions of alkanes, alkenes, aromatics, and heteroatoms. In general, these biotransformations are carried out by microbial cultures. Choloroperoxidase (CPO) has been employed for the enantioselective epoxidation of alkenes and olefins (Dexter et al., 1995).

Laccase is well documented with a mediator to oxidize certain aromatic compounds. This is called *mediated oxidation*. Laccase from the white-rot fungus *Trametes hirsuta* is employed for the oxidation of alkenes (Niku-Paavola and Viikari, 2000). This oxidation is a two-step process: (1) the enzyme catalyzes the oxidation of primary substrate, the mediator; and

(2) the oxidized mediator oxidizes the secondary substrate, the alkene. All alkenes are oxidized, and the extent of transformation depends on the alkene and the mediator. The highest degrees of conversion are obtained using hydroxybenzotriazole (HBT) as a mediator. Treatment at 20°C for 20 hours results in a 45 to 50% oxidation rate of α-pinene and, *cis*-2- and *cis*-3-hexenols and a 90 to 100% oxidation rate of linalool, geraniol, nerol, and cinnamyl alcohol. Other alkenes, such as allyl ether, *cis*-2-heptene, and cyclohexene, are oxidized less than 25%, even with all the mediators. The main reaction products of alkenes are aldehydes and ketones, but other products are also identified.

4.13   CYTOCHROME P450 ENZYME SYSTEMS

During the past decade, the role of cytochrome P450 enzymes in several complex fungal bioconversions has been established (van den Brink et al., 1998). Cytochrome P450–mediated bioconversions in filamentous fungi involve assimilation of long-chain alkanes and polycyclic aromatic hydrocarbons (PAHs). A well-studied example of alkane assimilation involving P450 process is carried out by species of *Candida* such as *C. apicola*, *C. maltosa*, and *C. tropicalis*. These bioconversions are terminal hydroxylation of *n*-alkanes and ω-hydroxylation of fatty acids. The monooxygenase system required for these reactions comprises several cytochrome P450s induced by alkanes (Seghezzi et al., 1991; Scheller et al., 1996). Comparable bioconversion reactions have also been examined in *Yarrowia lipolytica* (van Dyk et al., 1994). Certain fungal cytochrome P450–encoding genes for alkane assimilation have been identified and reviewed (van den Brink et al., 1998). Eight genes of a large cytochrome P450 gene family, *cyp52*, in species of *Candida* associated with *n*-alkane assimilation have been identified. Substrate specificity is different for each gene that reveals the capability of yeast to modify a range of different *n*-alkanes (Seghezzi et al., 1991; Scheller et al., 1996). Cytochrome P450 reductase (CPR)–encoding genes are also identified in *Aspergillus niger*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *C. tropicalis*, and *Candida maltosa*. Regulation of the expression of the fungal P450 *cyp52* system in *C. tropicalis* and *C. maltosa* has also been examined.

The presence of multiple *n*-alkane-inducible forms of cytochromes P450 (P450alk) that can catalyze terminal hydroxylation of *n*-alkanes in the assimilation pathway has been demonstrated in *C. maltosa* (Ohkuma et al., 1998). Eight structurally related P450alk genes are detected, and the function of four major isoforms of P450alk encoded by ALK1, ALK2, ALK3, and ALK5 genes were examined by sequential gene disruption. Ohtomo et al. (1996) reported the repression of four genes, ALK1, ALK2, ALK3, and ALK5, by glucose at the transcription level and transcriptionally activated in response to a peroxisome proliferator, clofibrate. A gene encoding cytochrome P450 involved in *n*-alkane assimilation is cloned from *Y. lipolytica* CX161–1B

(Iida et al., 1998). The expression of the P450 gene Y1ALK1 is induced by
n-tetradecane. A Y1ALK1 gene disruptant does not grow well on *n*-decane
but shows growth on longer-chain *n*-alkane such as hexadecane. The decane
utilization is restored by the introduction of Y1ALK1 on a plasmid to the
disruptant. This implies that the Y1ALK1 gene product is the major P450 to
assimilate short-chain alkanes such as decane and dodecane. The isolation of
more fungal P450 genes and their regulatory mechanisms and the cloning of
regulatory proteins are essential for an understanding of their role in alkane
metabolism. The P450 overproduction in fungi has a potential use in the
enhancement of bioremediation of oil contaminants in the environment.

4.14   ECONOMIC IMPORTANCE

The fermentation strategies for a potential biotechnological process on petro-
leum hydrocarbons as substrates are well known and have tremendous bio-
technology applications. These biotechnology applications are summarized
below.

4.14.1   Single-Cell Protein

Much of the work on single-cell protein (SCP) was accomplished in the
1970s and later abandoned due to marketing problems. Protein extracted
from petroleum by some yeasts and fungi has been described (Humphrey,
1970). *C. lipolytica* is used in the processing of alkane as a substrate for SCP
(Whiteworth, 1974). Of 67 potential yeasts, *C. tropicalis* and *Y. lipolytica* are
found to be excellent for SCP production on diesel oil as the sole source of
carbon. Maximum yield has been shown to occur for a diesel oil concentration
of 40 to 60 ml/l after 168 hours (Ashy and Abou-Zeid, 1982). A good source
of protein was achieved through fermentation of hydrocarbon-derived SCP,
and additional processing was required to remove undesirable components
(Scrimshaw, 1984). Cloning and recombinant DNA techniques can be used
to improve SCP production.

4.14.2   Surfactant Production

Fungi and yeasts produce a wide variety of surfactants, ranging from simple
fatty acids and phospholipids by filamentous species to complex polymers by
various yeasts. The extracellular phospholipids of *Cladosporium resinae* are
dodecanoic acid–substituted (Kan and Cooney, 1975). The most suitable
substrate for phospholipid formation by *Aspergillus* sp. is *n*-alkane C16
(Miyazima et al., 1985). Yeasts produce surfactants that exhibit highly active
emulsifying properties. Species of *Torulopsis* produce glycolipids that show
similarities to bacterial rhamnolipids. Acetyl-substituted disaccharide linked
to the hydroxyl function of a hydroxycarboxylic acid is found in sophorose

lipids of *Torulopsis bombicola* (Inoue and Ito, 1982). Sophorolipid production can be increased from 5 to 150 g/l in the presence of glucose and hexadecane (Linton, 1990). Species of *Candida* produce surfactants that are polysaccharide-lipid or lipopeptide in nature, produced either within the infrastructure of the cell wall or in the medium.

4.14.3   Metabolite Overproduction

Several yeasts and filamentous fungi produce a wide range of metabolities of industrial importance on hydrocarbons in the presence of optimum growth conditions. These products include organic acids, amino acids, antibiotics, sterols, and others. The use of products derived from hydrocarbons in the food industry must still obtain public approval.

Various strains of *C. lipolytica* are established to produce organic acids. Citric acid production exceeding 200 g/l is achieved by *C. lipolytica* grown on alkanes (Ikeno et al., 1975). Despite high production, the commercial production of citric acid employs *Aspergillus niger* and carbohydrate substrates. Several yeasts excrete small amounts of dicarboxylic acids during growth on alkanes due to ω-oxidation involving diterminal oxidation. By selection techniques and nonspecific mutagenesis, specific strains can result that show perturbance in β-oxidation. These strains accumulate dicarboxylic acids >100 g/l during growth on a mixture of alkane–acetate substrates. Even the isolation of mutants does not lead to complete removal of the activity of acyl-CoA oxidase. An industrial strain of *C. tropicalis* has been engineered for the production of long-chain dicarboxylic acids with a high degree of purity (Picataggio et al., 1992), and the schematic process has been illustrated by Lindley (1994). This process has the potential to replace the industrial chemical synthesis of long-chain dicarboxylic acids at an affordable price.

Peroxisomal enzymes can be employed in medical diagnosis tests (Tanaka et al., 1982). When cultivated at controlled phosphate concentrations and acidic pH, certain yeasts can accumulate various types of sugar alcohols. Peroxisomal enzymes can be overproduced. Fukui and Tanaka (1980) reported the production of both vitamin B_2 and B_6 and stimulation of cytochrome c and coenzyme Q to a high level on alkane media by yeasts. Several yeasts exhibit improved production of ergosterol during growth on alkanes. *Fusarium* sp. produced yields of 260 mg/l of ergosterol and 46 000 mg/l of extracellular alkaline protease (Nakao et al., 1973). *Paecilomyces carneus*, an alkane-assimilating fungus, has been employed to produce cephalosporin, a β-lactam family of antibiotics. Cephalosporin concentrations of 450 mg/l were obtained, but three forms of antibiotic were present in similar amounts (Kitano et al., 1976).

The future direction of long-term strategies of economic importance on alkane substrates is unclear. The potential of hydrocarbon-degrading yeasts and fungi has been largely ignored due to public unacceptance of the products. Harnessing the full potential of advances in molecular technology will

open the door for correct assessment of the economic importance of alkane-degrading yeasts and filamentous fungi in the future.

4.15   BIOTECHNOLOGY AND BIOENGINEERING

Advances in molecular biology and biotechnology have played a certain role in the fungal degradation of petroleum hydrocarbons. Some of this knowledge is summarized in the foregoing paragraphs regarding cytochrome P450 enzyme systems. Wang et al. (1999b) identified 5 acyl-coenzyme A (CoA) oxidase isozymes, Aox1-Aox5 in *Yarrowia lipolytica*, encoded by the POX1 to POX5 genes. The ACO3 gene, encoding one of the acyl-CoA oxidase isozymes, has been cloned and sequenced from *Y. lipolytica* (Wang et al., 1998). This gene allows narrow substrate specificity that is confirmed by expression in *Escherichia coli*. A genomic DNA clone encoding carnitine acetyltransferases, localized in two subcellular organelles, peroxisomes and mitochondria of *C. tropicalis*, has been isolated (Kawachi et al., 1996). Nucleotide and amino acid sequences have been analyzed. Kamasawa et al. (1996) presented immunoelectron microscopic observations on the localization of thiolase enzymes in *C. tropicalis*. This visual analysis is useful for the elucidation of a process of peroxisome proliferation and enzyme transport in a cell. The physiological functions of three thiolase isozymes in *C. tropicalis* have been evaluated by gene disruption (Kanayama et al., 1998). The role of peroxisomal heme in transport mechanism in *C. tropicalis* cells was examined by Soga et al. (1997). Yamamoto et al. (1995) described the purification of novel peroxisomal NADP-linked isocitrate dehydrogenase in *n*-alkane *C. tropicalis*–grown cells, which is effective in proliferation of peroxisomes. Such studies lead to an understanding of metabolic pathways related to mechanisms of degradation of hydrocarbons by fungi.

Different groups of microorganisms in the environment usually degrade petroleum hydrocarbons. The biodegradation is more rapid in temperate than in cold regions. Despite the retarding effect of low temperatures on biochemical reactions, these microorganisms grow at a similar rate to those living in the temperate regions. These microorganisms have developed various adaptations of structural changes in membranes, proteins, and enzymes. Thus, the use of these psychrophilic enzymes offer considerable potential in bioremediation processes (Gerday et al., 2000). In temperate regions, seasonal variations in temperature can reduce the effectiveness of microorganisms in degrading petroleum hydrocarbons. However, bioaugmentation and inoculation with specific cold-adapted microorganisms can improve the biodegradation of such oils. Due to the high catalytic efficiency of enzymes and specificity at low and moderate temperatures, these organisms have the potential for hydrocarbon bioremediation. Cold-adapted microorganisms, especially fungi, can open new horizons in the field of petroleum biotechnology and bioremediation.

4.16   CONCLUSIONS AND FUTURE PERSPECTIVES

Hydrocarbon degradation is site-specific and limited by the metabolic capabilities of hydrocarbon-degrading fungal populations and a wide range of environmental factors. The effectiveness of the process depends on identifying the rate-limiting factors and optimizing them in the feasibility studies. The various ecosystems, such as aquatic, freshwater, and terrestrial, present different challenges for fungal growth. Adapted fungal communities also play an important role in hydrocarbon degradation. Assessment methods can be employed to determine the existing conditions favorable for fungal growth, and respirometric tests can verify metabolically active fungal populations. Treatability studies performed with soils or slurries can be tested under several conditions, such as unmodified microcosm, nutrient-amended, and biologically inhibiting conditions, in order to collect useful data on the rate and extent of degradation. Process engineering variables are also to be integrated from each scale of observation for predicting the treatment. This approach suggests that the field data should be compared systematically to laboratory results to evaluate the success of the scale-up process.

Seeding the contaminated environments with exogenous fungi is unknown. The available knowledge on the value of seeding with exogenous microbes is not convincing enough to be considered a good approach for the bioremediation of petroleum hydrocarbons. Moreover, exogenous microbes have to compete with indigenous microbes to prove their effectiveness. Nutrient additions have been shown to overcome the critical rate-limiting factors. Addition of bulking agents (e.g., peat moss with bran flakes) produces the best growth, penetration, and enzyme activity of strains of *Bjerkandera adusta* in soil suitable for bioremediation (Meysami and Baheri, 2003). Genetically engineered fungal strains exhibiting improved capabilities for hydrocarbon metabolism can be seeded for the elimination of hydrocarbon pollutants. Mixed cultures of specific bacteria and fungi are also a potential target for seeding, due to different but complementary hydrocarbon-degrading capabilities. The seeding of mixed cultures has its own limitations and merits additional study.

REFERENCES

Ahearn, D.G., and S.P. Meyers (1972) The role of fungi in the decomposition of hydrocarbons in the marine environment. In: *Biodeterioration of Materials*, A.H. Walters and E.H. Hueck-van der Plas, eds. Wiley, New York, pp. 12–18.

Ahearn, D.G., and S.P. Meyers (1976) Fungal degradation of oil in the marine environment. In: *Recent Advances in Aquatic Mycology*, E.B. Gareth Jones, ed. Elek Science, London, pp. 125–134.

Ahearn, D.G., S.P. Meyers, and P.G. Standard (1971) The role of yeasts in the decomposition of oils in the marine environment. *Dev. Ind. Microbiol.* **12**: 126–134.

Alleman, B.C., and A. Leeson (1999) In situ bioremediation of petroleum hydrocarbon and other organic compounds. In: *Proceedings of the Fifth International In*

Situ and On-Site Bioremediation Symposium, Apr. 19–22, San Diego, CA. Battelle Press, Columbus, OH, Vol. 3, p. 588.

April, T.M., J.M. Foght, and R.S. Currah (2000) Hydrocarbon-degrading filamentous fungi isolated from flare pit soils in northern and western Canada. *Can. J. Microbiol.* **46**: 38–49.

Ashy, M.A., and A. Abou-Zeid (1982) Potentialities of yeasts in production of single cell proteins (SCP). *Z. Mikrobiol.* **137**: 387–394.

ASTM (1995) *Standard Guide for Risk-Based Corrective Action Applied at Petroleum Release Sites.* American Society for Testing and Materials, West Conshohocken, PA.

Atlas, R.M. (1995) Petroleum biodegradation and oil spill bioremediation. *Mar. Pollut. Bull.* **31**: 178–182.

Bailey, C.A., and M.E. May (1979) Evaluations of microbiological test kits for hydrocarbon fuel systems. *Appl. Environ. Microbiol.* **37**: 871–877.

Balba, M.T., N. Al-Awadhi, and R. Al-Daher (1998) Bioremediation of oil-contaminated soil: microbiological methods for feasibility assessment and field evaluation. *J. Microbiol. Methods* **32**: 155–164.

Bartha, R.R., and M. Atlas (1977) The microbiology of aquatic oil spills. *Adv. Appl. Microbiol.* **22**: 225–266.

Bartha, R., and Atlas, R.M. (1987) Transport and transformations of petroleum: biological processes. In: *Long-Term Effects of Petroleum on the Marine Environment*, D. Boesch and N. Rabalais, eds. Elsevier, London, pp. 287–341.

Bento, F.M., and C.C. Gaylarde (2001) Biodeterioration of stored diesel oil: studies in Brazil. *Int. Biodeterior. Biodegrad.* **47**: 107–112.

Boehm, P.D., G.S. Douglas, W.A. Burns, P.J. Mankiewicz, D.S. Page, and A.E. Bence (1997) Application of petroleum hydrocarbon chemical fingerprinting and allocation techniques after the *Exxon Valdez* oil spill. *Mar. Pollut. Bull.* **34**: 599–613.

Bossert, I., and R. Bartha (1984) The fate of petroleum in soil ecosystems. In: *Petroleum Microbiology*, R. Atlas, ed. Macmillan, New York, pp. 435–474.

Brakemeier, A., S. Lang, D. Wullbrandt, et al. (1995) Novel sophorose lipids from microbial conversion of 2-alkanols. *Biotechnol. Lett.* **17**: 1183–1188.

Cerniglia, C.E., and J.J. Perry (1973) Crude oil degradation by micro-organisms isolated from the marine environment. *Z. Allgern. Mikrobiol.* **13**: 299–306.

Chaineau, C.H., J. Morel, J. Dupont, E. Bury, and J. Oudot (1999) Comparison of the fuel oil biodegradation potential of hydrocarbon-assimilating microorganisms isolated from a temperate agricultural soil. *Sci. Total Environ.* **227**: 237–247.

Cofone, L., J.D. Walker, and J.J. Cooney (1973) Utilization of hydrocarbons by *Cladosporium resinae*. *J. Gen. Microbiol.* **86**: 243–246.

Colombo, J.C., M. Cabello, and A.M. Arambarri (1996) Biodegradation of aliphatic and aromatic hydrocarbons by natural soil microflora and pure cultures of imperfect and lignolitic fungi. *Environ. Pollut.* **94**: 355–362.

Cook, W.L., J.K. Massey, and D.G. Ahearn (1973) The degradation of crude oil by yeasts and its effects on *Lebistes reticulatus*. In: *The Microbial Degradation of Oil Pollutants*, D.G. Ahearn and S.P. Meyer, eds. Publication LSU-SG-73-0. Center for Wetland Resources, Louisiana State University, Baton Rouge, LA, pp. 279–282.

Cooney, J.J., and J.D. Walker (1973) Hydrocarbon utilization by *Cladosporium resinae*. In: *The Microbial Degradation of Oil Pollutants*, D.G. Ahearn and S.P. Meyer, eds. Publication LSU-SG-73-0. Center for Wetland Resources, Louisiana State University, Baton Rouge, LA, pp. 25–32.

Cooney, J.J., C. Siporin, and R.A. Smucker (1980) Physiological and cytological responses to hydrocarbons by the hydrocarbon-using fungus *Cladosporium resinae*. *Bot. Mar.* **23**: 227–232.

Cooney, J.J., S.A. Silver, and E.A. Beck (1985) Factors influencing hydrocarbon degradation in three freshwater lakes. *Microbiol. Ecol.* **11**: 127–137.

Davies, J.S., and D.W.S Westlake (1979) Crude oil utilization by fungi. *Can. J. Microbiol.* **25**: 146–156.

de Hoog, G.S., F.D. Marvin-Sikkema, G.A. Lahpoor, J.C. Gottschall, R.A. Prins, and E. Gueho (1994) Ecology and physiology of the emerging opportunistic fungi *Pseudallescheria boydii* and *Scedosporium prolificans*. *Mycoses* **37**: 71–78.

Dexter, A.F., F.J. Lakner, R.A. Campbell, and L.P. Hager (1995) Highly enantioselective epoxidation of 1,1-disubstituted alkenes catalyzed by chloroperoxidase. *J. Am. Chem. Soc.* **117**: 6412–6413.

Dix, N.J., and J. Webster (1995) The mycelium and substrates for growth. In: *Fungal Ecology*. Chapman & Hall, London, pp. 12–38.

Etkin, D.S., ed. (1997a) *Oil Spill Intelligence Report (Special)*. Cutter Information Corp., Arlington, MA, May.

Etkin, D.S., ed. (1997b) *Oil Spill Intelligence Report's White Paper Series*, Vol. 1(5). Cutter Information Corp., Arlington, MA, Sept.

Etkin, D.S., ed. (1997c) *Oil Spill Intelligence Report's White Paper Series*, Vol. 1(6). Cutter Information Corp., Arlington, MA, Oct.

Etkin, D.S., ed. (1998) *Oil Spill Intelligence Report's White Paper Series*, Vol. 2. Cutter Information Corp., Arlington, MA.

Faber, K. (1997) *Biotransformations in Organic Chemistry*, 3rd ed. Springer-Verlag, Berlin.

Fedorak, P.M., K.M. Semple, and D.W. Westlake (1984) Oil-degrading capabilities of yeasts and fungi from coastal marine environments. *Can. J. Microbiol.* **30**: 565–571.

Floodgate, G. (1984) The fate of petroleum in marine ecosystems. In: *Petroleum Microbiology*, R. Atlas, ed. Macmillan, New York, pp. 355–398.

Fuentes, F.A., J.W. Santo Domingo, and T.C. Hazen (1998) Survival of *Candida albicans* and *Pseudomonas aeruginosa* in oil polluted tropical coastal waters. *Water Res.* **32**: 2154–2170.

Fukui, S., and A. Tanaka (1979) Peroxisomes of alkane- and methanol-grown yeasts. *J. Appl. Biochem.* **50**: 133–157.

Fukui, S., and A. Tanaka (1980) Production of useful compounds from alkane media in Japan. *Adv. Biochem. Eng.* **17**: 1–36.

Gerday, C., M. Aittaleb, M. Bentahir, J.-P. Chessa, et al. (2000) Cold-adapted enzymes: from fundamentals to biotechnology. *Tibtech* **18**: 103–107.

Goma, G., A. Pareilleux, and G. Durand (1973) Specific hydrocarbon solubilization during growth of *Candida lipolytica*. *J. Ferment. Technol.* **51**: 616–618.

Harayama, S., H. Kishira, Y. Kasai, and K. Shutsubo (1999) Petroleum biodegradation in marine environments. *J. Mol. Microbiol. Biotechnol.* **1**: 63–70.

Head, I.M., and R.P. Swannell (1999) Bioremediation of petroleum hydrocarbon contaminants in marine habitats. *Curr. Opin. Microbiol.* **10**: 234–239.

Hoffmann, B., and H.J. Rehm (1976a) Degradation of long chain *n*-alkanes by Mucorales. I. Development of Mucorales on *n*-alkanes and some possible oxidation products. *Eur. J. Appl. Microbiol.* **3**: 19–30.

Hoffmann, B., and H.J. Rehm (1976b) Degradation of long chain *n*-alkanes by Mucorales. II. Extracellular oxidation products and degradation pathways. *Eur. J. Appl. Microbiol.* **3**: 31–41.

Hoffmann, B., E. Schulte, and H.J. Rehm (1977) Degradation of long chain *n*-alkanes by Mucorales. III. Glass capillary gas chromatography and mass spectroscopy of some extracellular oxidation products, especially isomeric esters, formed by *Mortierella isabelliana. Eur. J. Appl. Microbiol.* **3**: 273–281.

Humphrey, A.E. (1970) Microbial protein from petroleum. *Process Biochem.* **5**: 19–22.

Iida, T., A. Ohta, and M. Takagi (1998) Cloning and characterization of an *n*-alkane inducible cytochrome P450 gene essential for *n*-decane assimilation by *Yarrowia lipolytica. Yeast* **14**: 1387–1397.

Ijah, U.J.J. (1998) Studies on relative capabilities of bacterial and yeast isolates from tropical soil in degrading crude oil. *Waste Manag.* **18**: 293–299.

Ikeno, Y., M. Masuda, K. Tanno, I. Oomari, and N. Takahashi (1975) Citric acid production from various raw materials by yeasts. *J. Ferment. Technol.* **53**: 752–756.

Il'Chenko, A.P. (1984) Oxidase of higher alcohols in the yeast *Torulopsis candida* grown on hexadecane. *Mikrobiologiya* **53**: 589–597.

Inoue, S., and S. Ito (1982) Sophorose lipids from *Torulopsis bombicola* as microbial surfactants as alkane fermentations. *Biotechnol. Lett.* **4**: 3–8.

Jones, D.M., A.G. Douglas, R.J. Parkes, J. Taylor, W. Giger, and C. Schaffner (1983) The recognition of biodegraded petroleum-derived aromatic hydrocarbons in recent marine sediments. *Mar. Pollut. Bull.* **14**: 103–108.

Kamasawa, N., I. Ohtsuka, Y. Kamada, M. Ueda, A. Tanaka, and M. Osumi (1996) Immunoelectron microscopic observation of the behaviors of peroxisomal enzymes inducibly synthesized in an *n*-alkane-utilizable yeast, *Candida tropicalis. Cell Struct. Funct.* **21**: 117–122.

Kan, C.M.L., and J.J. Cooney (1975) Phospholipids of *Cladosporium resinae* cultured on glucose or on *n*-alkanes. *Can. J. Microbiol.* **21**: 1205–1210.

Kanayama, N., M. Ueda, H. Atomi, and A. Tanaka (1998) Genetic evaluation of physiological functions of thiolase isoenzymes in the *n*-alkane-assimilating yeast *Candida tropicalis. J. Bacteriol.* **180**: 690–698.

Kaplan, I.R., Y. Galperin, S.T. Lu, and R.P. Lee (1997) Forensic environmental geochemistry: differentiation of fuel-types, their sources and release time**.** *Org. Geochem.* **27**: 289–317.

Kappeli, O., and A. Fiechter (1981) Properties of hexadecane uptake by *Candida tropicalis. Curr. Microbiol.* **6**: 21–26.

Kawachi, H., H. Atomi, M. Ueda, and A. Tanaka (1996) Peroxisomal and mitochondrial carnitine acetyltransferases of *n*-alkane-assimilating yeast *Candida tropicalis*: analysis of gene structure and translation products. *Eur. J. Biochem.* **238**: 845–852.

Kirk, P.W. (1983) Direct enumeration of marine arenicolous fungi. *Mycologia* **75**: 670–682.

Kirk, P.W., and A.S. Gordon (1988) Hydrocarbon degradation by filamentous marine higher fungi. *Mycologia* **80**: 776–782.

Kitano, K., K. Kintaka, S. Suzuki, K. Katamoto, K. Nara, and Y. Nakao (1976) Screening of microorganisms capable of producing β-lactam antibiotics from *n*-paraffins. *J. Ferment. Technol.* **54**: 683–695.

Kohlmeyer, J., and E. Kohlmeyer (1979) *Marine Mycology: The Higher Fungi*. Academic Press, New York.

Komagata, K., T. Nakase, and N. Katsuya (1964) Assimilation of hydrocarbons by yeasts: preliminary screening. *J. Gen. Microbiol.* (*Tokyo*) **10**: 313–321.

Krahn, M.M., G.M. Ylitalo, J. Buzitis, S. Chan, and U. Varanasi (1993) Rapid high-performance liquid chromatographic methods that screen for aromatic compounds in environmental samples. *J. Chromatogr. A* **642**: 15–32.

Kram, M. (1998) Use of SCAPS petroleum hydrocarbon sensor technology for real-time indirect DNAPL detection. *J. Soil Contam.* **7**: 73–86.

Kremer, S., and H. Anke (1997) Fungi in bioremediation. In: *Fungal Biotechnology*, T. Anke, ed. Chapman & Hall, Weinheim, Germany, pp. 275–295.

Kulichevskaya, I.S., V.S. Guzev, and N.S. Panikov (1995) Population dynamics of hydrocarbon-oxidizing yeasts introduced into oil-contaminated soil. *Microbiology* **64**: 569–573.

Laborda, F., A. Moreno, A.M. Pedregosa, and M.M. Martin (1996) Screening and selection of hydrocarbon degrading moulds regarding bioremediation. *Int. Biodeterior. Biodegrad.* **37**: 245.

Lambert, P., M. Fingas, and M. Goldthorp (2001) An evaluation of field total petroleum hydrocarbon (TPH) systems. *J. Hazard. Mater.* **83**: 65–81.

Leahy, J.G., and R.R. Colwell (1990) Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev.* **54**: 305–315.

Lindley, N.D. (1991) Hydrocarbon-degrading yeasts and filamentous fungi of biotechnological importance. In: *Handbook of Applied Mycology*, Vol. 4, *Biotechnology*, D.K. Arora, K.G. Mukerji, and R.P. Elander, eds. Marcel Dekker, New York, pp. 905–929.

Lindley, N.D. (1994) Bioconversion and biodegradation of aliphatic hydrocarbons. *Can. J. Bot.* **73**(Suppl. 1): S1034–S1042.

Lindley, N.D., and M.T. Heydeman (1986) Mechanism of dodecane uptake by whole cells of *Cladosporium resinae*. *J. Gen. Microbiol.* **132**: 751–756.

Linton, J.D. (1990) Potential of dual substrates for biomass and metabolite production. In: *Mixed and Substrates and Feedstocks: Proceedings of the Discussion Meeting of the European Federation of Biotechnology*, Nov. 25–26, 1988, Weinfelden, Switzerland, G. Hamar, T. Egli, and M. Snozzi, eds. Hartnung-Gorre, Konstanz, Switzerland, pp. 33–44.

Llanos, C., and A. Kjoller (1976) Changes in the flora of soil fungi following oil waste application. *Oikos* **27**: 377–382.

Lundanes, E., and T. Greibrokk (1994) Separation of fuels, heavy fractions, and crude oils into compound classes: a review. *J. High Resolut. Chromatogr.* **17**: 197–202.

Margesin, R., and F. Schinner (1997) Effect of temperature on oil degradation by a psychrotrophic yeast in liquid culture and in soil. *FEMS Microbiol. Ecol.* **24**: 243–249.

McCutcheon, S.C., and J.L. Schnoor (2003) *Phytoremediation: Transformation and Control of Contaminants.* Wiley-Interscience, Hoboken, NJ.

Meysami, P., and H. Baheri (2003) Pre-screening of fungi and bulking agents for contaminated soil bioremediation. *Adv. Environ. Res.* **7**: 881–887.

Middelhoven, W.J., I.M. de Jong, and M. de Winter (1991) *Arxula adeninivorans*, a yeast assimilating many nitrogenous and aromatic compounds. *Antonie Leeuwenhoek* **59**: 129–137.

Middelhoven, W.J., M. Koorevaar, and G.W. Schuur (1992) Degradation of benzene compounds by yeast in acidic soil. *Plant Soil* **145**: 37–43.

Middelhoven, J.W., G. Scorzetti, and J.W. Fell (2000) *Trichosporon veenhuisii* sp. nov., an alkane-assimilating anamorphic basidiomycetous yeast. *Int. J. Syst. Evol. Microbiol.* **50**: 381–387.

Miyazima, M., M. Iida, and H. Iizuka (1985) Phospholipid derived from hydrocarbons by fungi. *J. Ferment. Technol.* **63**: 219–224.

Morselli, L., L. Setti, A. Iannuccilli, S. Maly, G. Dinelli, and G. Quattroni (1999) Supercritical fluid extraction for the determination of petroleum hydrocarbons in soil. *J. Chromatogr. A* **845**: 357–363.

Nair, S.A., and P.A. Lokabharathi (1977) Degradation of hydrocarbons by the fungus, *Fusarium* sp. *Indian J. Mar. Sci.* **6**: 173–175.

Nakao, Y., M. Suzuki, M. Kuno, and K. Maejima (1973) Production of alkaline protease from *n*-alkanes by a kabicidin resistant mutant strain of *Fusarium* sp. *Agric. Biol. Chem.* **37**: 1223–1224.

National Research Council (1994) *Alternatives for Ground-Water Clean-Up.* National Academy Press, Washington, DC.

Neihof, R., and M. May (1983) Microbial and particulate contamination in fuel tanks on naval ships. *Int. Biodeterior. Bull.* **19**: 59–68.

Niku-Paavola, M.-L., and L. Viikari (2000) Enzymatic oxidation of alkenes. *J. Mol. Catal. B* **10**: 435–444.

Norris, R.D., et al. (1994) *Handbook of Bioremediation.* Lewis Publishers, Boca Raton, FL.

Ohkuma, M., T. Zimmer, T. Iida, W.H. Schunck, A. Ohta, and M. Takagi (1998) Isozyme function of *n*-alkane-inducible cytochrome P450 in *Candida maltosa* revealed by sequential gene disruption. *J. Biol. Chem.* **273**: 3948–3953.

Ohtomo, R., K. Kobayashi, S. Muraoka, M. Ohkuma, A. Ohta, and M. Takagi (1996) Peroxisome proliferators activate cytochrome P450 genes in an alkane-assimilating yeast, *Candida maltosa. Biochem. Biophys. Res. Commun.* **222**: 790–793.

Okpokwasili, G.C., and S.C. Amanchukwu (1988) Petroleum hydrocarbon degradation by *Candida* species. *Environ. Int.* **14**: 243–247.

Oudot, J., J. Dupont, S. Haloui, and M.F. Roquebert (1993) Biodegradation potential of hydrocarbon-assimilating tropical fungi. *Soil Biol. Biochem.* **25**: 1167–1173.

Pelz, B.F., and H.J. Rehm (1973) Terminale und subterminale Oxidation von *n*-Alkanen durch Schimmelpilze. *Arch. Mikrobiol.* **92**: 153–170.

Perry, J.J. (1979) Microbial co-oxidations involving hydrocarbons. *Microbiol. Rev.* **43**: 59–72.

Pezeshki, S.R., M.W. Hester, Q. Lin, and J.A. Nyman (2000) The effect of oil spill and clean-up on dominant US Gulf coast marsh macrophytes: a review. *Environ. Pollut.* **108**: 129–139.

Picataggio, S., T. Rohrer, K. Deanda, D. Lanning, R. Reynolds, J. Mielenz, and L.D. Eirich (1992) Metabolic engineering of *Candida tropicalis* for the production of long-chain dicarboxylic acids. *Bio/Technol.* **10**: 894–898.

Pinholt, Y., S. Struwe, and A. Kjoller (1979) Microbial changes during oil decomposition in soil. *Holarct. Ecol.* **2**: 195–200.

Prenafeta-Boldu, F.X., D.M.A. Luykx, J. Vervoort, and J.A.M. de Bont (2001) Fungal metabolism of toluene: monitoring of fluorinated analogs by ^{19}F nuclear magnetic resonance spectroscopy. *Appl. Environ. Microbiol.* **67**: 1030–1034.

Prenafeta-Boldu, F.X., J. Vervoort, J.T.C. Grotenhuis, and J.W. van Groenestijn (2002) Substrate interactions during the biodegradation of benzene, toluene, ethylbenzene, and xylene (BTEX) hydrocarbons by the fungus *Cladophialophora* sp. strain T1. *Appl. Environ. Microbiol.* **68**: 2660–2665.

Prince, R.C. (1993) Petroleum spill bioremediation in marine environments. *Crit. Rev. Microbiol.* **19**: 217–242.

Qi, B., W.M. Moe, and K.A. Kinney (2002) Biodegradation of volatile organic compounds by five fungal species. *Appl. Microbiol. Biotechnol.* **58**: 684–689.

Reddy, C.M., and J.G. Quinn (2001) The *North Cape* oil spill: hydrocarbons in Rhode Island coastal waters and Point Judith Pond. *Mar. Environ. Res.* **52**: 445–461.

Rudd, L.E., J.J. Perry, V.S. Houk, R.W. Williams, and L.D. Claxton (1996) Changes in mutagenicity during crude oil degradation by fungi. *Biodegradation* **7**: 335–343.

Sauer, T.C., and P.D. Boehm (1995) *Technical Report Series 95-032*. Marine Spill Response Corp., Washington, DC.

Scheller, U., T. Zimmer, E. Kargel, and W.-H. Schunck (1996) Characterization of the *n*-alkane and fatty acid hydroxylating cytochrome P450 forms 52A3 and 52A4. *Arch. Biochem. Biophys.* **328**: 245–254.

Schmitz, C., I. Goebel, S. Wagner, A. Vomberg, and U. Klinner (2000) Competition between *n*-alkane-assimilating yeasts and bacteria during colonization of sandy soil microcosms. *Appl. Microbiol. Biotechnol.* **54**: 126–132.

Scrimshaw, N.S. (1984) Acceptance of single-cell protein for human food applications. In: *Comprehensive Biotechnology*, C.W. Robinson and J.A. Howell, eds. Pergamon Press, Oxford, Vol. 4, pp. 673–684.

Seghezzi, W., D. Sanglard, and A. Fiechter (1991) Characterization of a second alkane-inducible cytochrome P450-encoding gene, Cyp52A2, from *Candida tropicalis*. *Gene* **106**: 51–60.

Sherry, J.P. (1984) The impact of oil and oil dispersant mixtures on fungi in freshwater ponds. *Sci. Total Environ.* **35**: 149–167.

Singer, M.E., and W.R. Finnerty (1984) Microbial metabolism of straight-chain and branched alkanes. In: *Petroleum Microbiology*, R.M. Atlas, ed. Macmillan, New York, pp. 1–60.

Smith, R.V., and J.P. Rosazza (1974) Microbial models of mammalian metabolism: aromatic hydroxylation. *Arch. Biochem. Biophys.* **161**: 551–558.

Snellman, E.A., R.P. Collins, and J.C. Cooke (1988) Utilization of fuel oil by fungi isolated from oceanic tar bulls. *Lett. Appl. Microbiol.* **6**: 105–107.

Soga, O., H. Kinoshita, M. Ueda, and A. Tanaka (1997) Evaluation of peroxisomal heme in yeast. *J. Biochem.* **121**: 25–28.

Sturman, P.J., P.S. Stewart, A.B. Cunningham, E.J. Bouwer, and J.H. Wolfram (1995) Engineering scale-up of in situ bioremediation processes: a review. *J. Contam. Hydrol.* **19**: 171–203.

Tanaka, A., M. Osumi, and S. Fukui (1982) Peroxisomes of alkane-grown yeasts: fundamental and practical aspects. *Ann. NY Acad. Sci.* **386**: 183–199.

Tracy, A. (1997) The use of immunoassay technology to delineate petroleum hydrocarbon contamination. *Fuel Energy Abstr.* **38**: 53.

Uchio, R., and J. Shiio (1972) Tetradecane-1,14-dicarboxylic acid production from *n*-hexadecane by *Candida cloacae. Agric. Biol. Chem.* **36**: 1289–1297.

van den Brink, H(J).M., R.F.M. van Gorcom, C.A.M.J.J. van den Hondel, and P.J. Punt (1998) Cytochrome P450 enzyme systems in fungi. *Fungal Genet. Biol.* **23**: 1–17.

van Dyk, M.S., J.L.F. Kock, and A. Botha (1994) Hydroxy long-chain acids in fungi. *World J. Microbiol. Biotechnol.* **10**: 495–504.

Walker, J.D., and R.R. Colwell (1974) Microbial degradation of model petroleum at low temperatures. *Microb. Ecol.* **1**: 63–95.

Walker, J.D., and R.R. Colwell (1976) Enumeration of petroleum-degrading microorganisms. *Appl. Environ. Microbiol.* **31**: 198–207.

Wang, Z., and M. Fingas (1997) Development in the analysis of petroleum hydrocarbons in oils, petroleum products and oil-spill related environmental samples by gas chromatography. *J. Chromatogr. A* **774**: 51–78.

Wang, H.J., A. Le Clainche, M.T. Le Dall, Y. Wache, Y. Pagot, J.M. Belin, C. Gaillardin, and J.M. Nicaud (1998) Cloning and characterization of the peroxisomal acyl CoA oxidase ACO3 gene from the alkane-utilizing yeast *Yarrowia lipolytica. Yeast* **14**: 1373–1386.

Wang, Z., M. Fingas, and D.S. Page (1999a) Oil spill identification. *J. Chromatogr. A* **843**: 369–411.

Wang, H.J., M.T. Le Dall, Y. Wache, C. Laroche, and J.M. Belin, C. Gaillardin, and J.-M. Nicaud, (1999b) Evaluation of acyl coenzyme A oxidase (Aox) isozyme function in the *n*-alkane-assimilating yeast *Yarrowia lipolytica. J. Bacteriol.* **181**: 5140–5148.

Westlake, D.W.S., A.M. Jobson, and F.D. Cook (1978) Degradation of oil in soil of the boreal region of the North-West territories. *Can. J. Microbiol.* **24**: 254–260.

Whiteworth, D.A. (1974) Hydrocarbon fermentation: protein and enzyme solubilization from *Candida lipolytica* using an industrial homogenizer. *Biotechnol. Bioeng.* **16**: 1399–1406.

Whittaker, M., S.J.T. Pollard, and T.E. Fallick (1995) Characterization of refractory wastes at heavy oil-contaminated sites: a review of conventional and novel analytical methods. *Environ. Technol*, **16**: 1009–1033.

Yadav, J.S., and C.A. Reddy (1993) Degradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **59**: 756–762.

Yamada-Onodera, K., H. Mukumoto, Y. Katsuyama, and Y. Tani (2002) Degradation of long-chain alkanes by a polyethylene-degrading fungus, *Penicillium simplicissimum* YK. *Enzyme Microb. Technol.* **30**: 828–831.

Yamamoto, S., H. Atomi, M. Ueda, and A. Tanaka (1995) Novel NADP-linked isocitrate dehydrogenase present in peroxisomes of *n*-alkane-utilizing yeast, *Candida tropicalis*: comparison with mitochondrial NAD-linked isocitrate dehydrogenase. *Arch. Microbiol.* **163**: 104–111.

Yateem, A., M.T. Balba, and N. Al-Awadhi (1998) White rot fungi and their role in remediating oil-contaminated soil. *Environ. Int.* **24**: 181–187.

Yi, Z.H., and H.J. Rehm (1982) Degradation pathways from n-tridecane to α,ω tridecanedioic acid in a mutant of *Candida tropicalis*. *Appl. Microbiol. Biotechnol.* **15**: 144–146.

5

FUNGAL DEGRADATION OF POLYCHLORINATED BIPHENYLS AND DIOXINS

5.1 INTRODUCTION

Polychlorinated biphenyls are a family of compounds containing a biphenyl molecule that is chlorinated to form a number of possible congeners. Polychlorinated biphenyls (PCBs) differ in the number and distribution of chlorine (1 to 10) on the biphenyl nucleus. PCBs exhibit the chemical formula $C_{12}H_{10-n}Cl_n$. They are used extensively in a wide variety of commercial applications in industrial processes and products because of excellent chemical stability and electrical insulating properties. Their applications range from the manufacturing of adhesives, printing, surface coatings, textiles, plastics, lubricants, and cooling systems to dielectric fluids (capacitors and transformers), hydraulic fluids, and fire retardants. PCBs were synthesized from the 1920s to 1978 in Japan, Europe, and the United States. Accidental spills, leaking of PCBs from electric transformers, and improper practices to dispose of industrial PCB wastes contributed contamination of various media, ranging from soils and sediments to rivers and streams.

PCBs are among the most widespread and recalcitrant contaminants and are present in abiotic and biotic environments throughout the world. These chemicals are known to persist for several decades, and this persistence can be attributed to the inability of microorganisms to metabolize these compounds. Their toxicity and role in disrupting the endocrine system are well known. Kimbrough (1995) described the impact of PCBs on human health. Research in the past two decades has thoroughly documented the fate and

behavior of PCBs in global ecosystems. Due to their carcinogenic, mutagenic, teratogenic, and other toxic properties, PCBs have been phased out worldwide because of their applications during the 1970s.

In 1976, the U.S. Congress enacted the Toxics Substances Control Act (TSCA), regulating the manufacture, processing, distribution in commerce, and use of PCBs (40 CFR 761 et seq.). The PCB Spill Cleanup Policy, regulated by Subpart G of 40 CFR 761, happened after the effective date (May 4, 1987). PCBs are also regulated under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), governed by the U.S. Environmental Protection Agency. Cleanup of widespread contamination at current and former industrial sites continues to take place under the Superfund. Robinson and Lenn (1994) have described some aspects of environmental legislation related to contaminated land and water due to PCBs.

5.2 NOMENCLATURE

According to the IUPAC, PCBs consist of two rings, one ring labeled clockwise and the other anticlockwise, with the numbering of subsequent positions in the molecule. There are 209 possible congeners of PCBs based on differences in the number and arrangement of chlorine substituents. The nomenclature of the numbering system has been adopted for these chemicals. Approximately 150 congeners are known to exist in the environment (Yadav et al., 1995). Industrial mixtures such as the Aroclor series (United States), Kaneclors (Japan), Sovols (former USSR), Clophen (Germany), Phenoclor and Pyralene (France), and Fenchlor (Italy) are widely produced and used on the basis of restricted chlorination of biphenyl. Aqueous solubility and volatility decrease with additional substitution. The order of chlorination decreases as follows: Aroclor 1242 > 1248 > 1260. They also exhibit differences in physical appearance [e.g., clear liquids (Aroclor 1242–1248) to lightcolored oils (Aroclor 1254–1262), to off-white powder (Aroclor 1268)].

5.3 BIOACCUMULATION AND TOXICITY

These compounds are difficult to degrade, are not easily metabolized, and tend to bioaccumulate in fatty tissue occupying higher trophic levels. Several factors are responsible for PCB bioaccumulation (Hamdy and Gooch, 1986). These include the concentration in the environment, duration of exposure, contaminant solubility, species age, weight, feeding habits and lipid level, variations in trophic level, and adsorption. Moreover, studies on bioaccumulation and degradation by bacteria and uptake of PCBs by yeasts, fungi, phytoplanktons, and marine diatoms are summarized. The uptake and bioconcentration in aquatic invertebrates and studies on the metabolism of PCBs by birds and mammals are also reviewed.

All Aroclors inhibit the growth of *Saccharomyces cerevisiae* when employed in fermentable media (Tejedor et al., 1979). Cole et al. (1979) calculated bioconcentration factors of 307 ± 63 for biphenyl and 1547 for 4-chlorobiphenyl by *Rhodotorula rubra*. Certain diatoms are also found to concentrate PCBs (Keil et al., 1971; Biggs et al., 1980). Boyle et al. (1992) estimated the amount of PCBs in the environment of different media. The cleanup of hot spots of PCB contamination is an important issue. PCBs are known to bind covalently to deoxyribonucleic acid (DNA), both in vivo and in vitro. Huang and Gibson (1992) reported the relation of acute toxicity to coplaner configuration of the chlorinated biphenyls. Various factors [e.g., the log *P* value of the congeners and the steric effect coefficients (SECs)] contribute to the process of bioaccumulation. Hydrophobicity is responsible for the accumulation and fate of PCBs in biological systems. The central issue is to discover the potential of microorganisms to detoxify these compounds.

5.4   ALTERNATIVE PCB REMEDIATION TECHNOLOGIES

Due to their ubiquitous nature, the development of PCB remediation technologies is in progress. Current knowledge of PCB remediation technologies is far from complete. The cleanup goal must be finalized by regulatory agencies before the implementation of remediation technology. Moreover, the ability to attain the cleanup goal in a given contaminated site by technology is also considered. The main purpose of establishing a cleanup goal is to protect human health and the environment. Many hazardous waste treatment technologies are well recognized in treating such wastes. Alternative PCB remediation technologies in various media are listed in Table 5.1.

5.5   ANALYSIS OF POLYCHLORINATED BIPHENYLS

During PCB biodegradation, chlorides are generally released in the system and are measured. Several methods are known for the detection of chlorides, ranging from simple colorimetric to complex chromatographic methods. Robinson and Lenn (1994) discussed several methods for the detection of chlorides, the degree of sensitivity, and the corresponding references. These methods include atomic adsorption, colorimetric, endpoint titration with silver ions, modified Mohr's titration, x-ray fluorescence spectrophotometry, ion chromatography, and ion chromatography with an electric conductivity detector. These methods are also used to elucidate metabolic pathways when used in conjunction with GC or HPLC.

At present, gas chromatography (GC) is the most common method of PCB analysis. However, the detectors are used along with GC to enhance its resolution. Flame ionization detectors (FIDs) and electron capture detectors

TABLE 5.1   Polychlorinated Biphenyl Remediation Technologies

Technology	Medium	Reference
Incineration	Soil, liquid	OHM, 1995
In situ vitrification	Soil	Hansen, 1998
Landfill cap system	Soil	USEPA, 1999
Chemical dehalogenation		
Alkali metal polyethylene glycolate and base-catalyzed dechlorination	Soil, liquid	Rahuman et al., 2000
Fe^{3+} ions, H_2O_2, and UV light		Pignatello and Guadalupe, 1994
Solvent extraction	Soil	USEPA, 1998
Chemical catalytic techniques		
SACRED (Samarium diiodide–catalyzed reductive dechlorination)	Soil, aqueous	Jackman et al., 1999
Titanocene dichloride process	Soil	Wright et al., 1996
Fenton's reagent	Aqueous	Sedlak and Andren, 1991
Photochemical	Liquid	Legrini et al., 1993
Bioremediation		
Aerobic bioremediation	Soil, aqueous	Rojas-Avelizapa et al., 1999; Pieper, 2005
Anaerobic bioremediation	Soil, sediments	Bedard and Quensen, 1995 Robinson and Lenn, 1994
Phytoremediation	Soil	Harms et al., 2003
Enzyme treatment		
Horseradish peroxidase with hydrogen peroxide	Aqueous	Koller et al., 2000

(ECDs) are employed along with GC on a regular basis. High-performance liquid chromatography (HPLC) has also been used along with ultraviolet (UV) detectors in the detection of PCBs. Mass spectrometer (MS) is also employed in conjunction with GC for individual PCB congeners that do not coelute. Erickson (1997) has described the analytical chemistry of PCBs.

The GC-ECD and capillary column are employed for the estimation of PCBs in soils by white-rot fungi, methylotrophic and hydrocarbon-utilizing yeasts, and bacteria (Sasek et al., 1993; Kubatova et al., 2001). The potential to transform di-, tetra-, and hexachlorinated PCBs using *Phanerochaete chrysosporium* has been studied by HPLC, GC-MS, and TLC and autoradiography (Dietrich et al., 1995). Cloete and Celliers (1999) quantified the remaining amount of Aroclor 1254 in the cultures by GC-ECD. Arochlor degradation is analyzed by GC-ECD in a fused silica capillary column at 70 eV in a full-scan mode. A GC-MS is also employed for the analysis of a wide range of degradation products of PCB 9 using the white-rot fungus

Trametes multicolor (Koller et al., 2000). The PCB extract is evaporated in a rotary evaporator, purified in a florisil column, and analyzed by HPLC (Fernandez-Sanchez et al., 1999; Ruiz-Aguilar et al., 2002).

5.6 BIOAVAILABILITY OF POLYCHLORINATED BIPHENYLS

Bioavailability is used to interpret the behavior of chemical constituents in the presence of microorganisms and plants in soils, sediments, and water. Nine definitions of bioavailability and its significance for understanding the fate of chemical constituents have been compiled (Madsen, 2003). Based on the persistence of PCBs in nature, seven hypothesized mechanisms were put forth in which bioavailability influences bioremediation. These include sorption, non-aqueous-phase liquid (NAPL) partitioning, micropores, nanopores, organic complexation, insolubility, and absorption into native organic matter. Bioavailability of persistent organic compounds in the environment has also been described (Alexander, 1997; Baveye and Bladon, 1999). Methods for measurement of the bioavailability of PCBs in soils or sediments are unknown at the present time. There is a three-way interaction between microorganisms, PCBs, and a geochemical matrix. Soil or sediment chemistry has a significant impact on sorption and PCB bioavailability. Knowledge of how to estimate the rate of release of PCBs and its kinetics from soils and sediments is not available. Knowledge of the influence of various physicochemical parameters (diffusion coefficients, hydrodynamics, sorption coefficients, matrix structure) and processes (dissolution, desorption) on the persistence of these compounds can be gained to predict bioavailability in various physical states. A systematic protocol for determining the bioavailability and biokinetics of organic pollutants in soil slurry, compacted bioreactors, and intact soil systems (Tabak and Govind, 1997) and the elucidation of fundamental mechanisms of bioavailability (Voice et al., 1997) have been developed. Quantifying bioavailability is subject to mass transfer limitations (Bosma et al., 1997). Chemical and biochemical reactions to depict certain interactions, processes, and mechanisms of action due to bioavailability have not been elucidated. Zeddel et al. (1993) reported a lower rate of PCB degradation due to lowered bioavailability. The important requirements include the homogenization process and proper contact of mycelia of fungi and soil aggregates. Conventional mixing cannot degrade the higher chlorinated PCBs.

   The use of *pollutant carriers* such as surfactant or organic cosolvents is useful for enhancing the solubility of PCBs. The surfactant can be toxic to microbial populations. The addition of surfactant to an aqueous system results in the formation of micelles when the aqueous surfactant concentration is above the critical micelle concentration (CMC) and the biodegradation is modeled via the Monod equation (Guha and Jaffe, 1996; Guha et al., 1998). Layton et al. (1998) studied an integrated bioremediation process linking surfactant washing of PCBs from soils, followed by bacterial aerobic degrada-

tion. The residual PCBs are deposited on a solid carrier, resulting in 90% removal of PCBs.

5.7   FUNGAL DEGRADATION OF POLYCHLORINATED BIPHENYLS

5.7.1   Filamentous Fungi

PCBs were first identified in 1966 (Jensen, 1966) as a new environmental contaminant of public and regulatory concern (Colvin and Nelson, 1990). Soon after this discovery, their persistence in different matrices has given birth to various methods of treatment. Recently, the popularity of conventional technologies of incineration and landfarming has declined due to their transfer of contamination from one medium to another (e.g., land to air and land to land). Due to the cost and demerits of technologies listed above, researchers began to screen different types of microorganisms (fungi, yeasts, bacteria, and algae) to explore new frontiers of PCB degradation. Degradation of PCBs and formation of metabolic products by filamentous fungi is noted in Table 5.2.

Fungal species belonging to several genera have the ability to grow on biphenyl and produce 2- and 4-hydroxybiphenyls and 4,4′-dihydroxybiphenyl (Smith and Rosazza, 1974). Murado et al. (1976) noted interactions between PCBs and soil fungi. A concentration range 5 to 50 μg/ml of Aroclor 1254 in the culture medium decreased the dry weight of *Aspergillus flavus*. The negative effect was highly pronounced in Aroclor 1232 and lowest in Aroclor 1260. PCB mixtures containing 32 to 60% chlorine by weight were not metabolized by *A. flavus*. Resting cells of Sabouraud-grown *Aspergillus niger* degraded the lower chlorinated PCBs present in Clophen A 30 (a technical PCB preparation averaging 42% chlorine by weight) using a replacement culture technique (Dmochewitz and Ballschmiter, 1988). Three isomers of hydroxylated trichlorobiphenyls in the ratio 15:3:1 and traces of dichloro- and trichlorobenzoic acids were detected. The principle of *para*-recalcitrance was established. *Cunninghamella elegans* grown on Sabouraud dextrose broth can hydroxylate biphenyl, giving rise to 2-, 3- and 4-hydroxybiphenyls and 4,4′-dihydroxybiphenyl as the major metabolites (Dodge et al., 1979). A tentatively identified compound such as 2,4′-dihydroxybiphenyl was also produced. This fungus also transformed 4-hydroxybiphenyl and 2-hydroxybiphenyl to 4,4′-dihydroxybiphenyl and 2,5-dihydroxybiphenyl, respectively. A survey of 11 fungal species shows more or less similar hydroxylation of biphenyl by two species comparable to *C. elegans*. In addition, 44% of metabolites produced by *C. elegans* are the water-soluble glucuronides and sulfate conjugates. Pathways proposed for the initial transformation of biphenyl by *C. elegans* are also known.

Ten fungi produced 4-hydroxybiphenyl, and seven of the 10 released 4,4′-dihydroxybiphenyl on biphenyl in a study by Schwartz et al. (1980).

TABLE 5.2 Fungal (Filamentous Fungi and Yeasts) Degradation of Polychlorinated Biphenyls

Filamentous Fungus/ Yeast	PCB Conc.	Medium	Degradation Product(s)	Duration (hours/days)	Reference
Aspergillus niger	Clophen A 30, 21.850 μg	Sabouraud	Hydroxylated trichlorobiphenyls, dichloro- and trichlorobenzoic acids	19 d	Dmochewitz and Ballschmiter, 1988
Cunninghamella elegans	Biphenyl, 2 mg/ml 4-Hydroxybiphenyl 2-Hydroxybiphenyl	Sabouraud dextrose broth	2-, 3-, and 4-Hydroxybiphenyls, 4,4′-dihydroxybiphenyl 4,4′-Dihydroxybiphenyl 2,5-Dihydroxybiphenyl	48 h	Dodge et al., 1979
Ten fungi Seven of 10 fungi	Biphenyl, 3.5 g/l	Five types	4-Hydroxybiphenyl 4,4′-Dihydroxybiphenyl	14 d	Schwartz et al., 1980
Cunninghamella echinulata Helicostylum piriforme Aspergillus parasiticus	Biphenyl, 375 mg	Soybean meal–glucose medium	4-Hydroxybiphenyl 2-Hydroxybiphenyl 4,4′-Dihydroxybiphenyl	72 h	Smith et al., 1980
Trichosporon mucoides	Biphenyl (as diethyl ether solution), 250 μg/ml	Glucose- or phenol-grown cells	Mono-, di-, and trihydroxylated products hydroxylated on one or both rings	48 h	Sietmann et al., 2001
Trichosporon beigelii	Diphenyl ether, 3–15 μl	Mineral salt medium with glucose and vitamin	2-, 3-, and 4-Hydroxydiphenyl ethers, 3,4-dihydroxydiphenyl ether, 6-carboxy-4-phenoxy-2-pyrone	24 h	Schauer et al., 1995
Debaryomyces vanrijae	Biphenyl, 500 mg/l 2-Hydroxybiphenyl, 125 mg/l 4-Hydroxybiphenyl, 125 mg/l	Mineral salt medium with glucose Medium with tetradecane	4-Hydroxybiphenyl and 2-hydroxybiphenyl 2,5-Dihydroxybiphenyl 3,4-Dihydroxybiphenyl, 4-phenylmuconolactone	70 h	Lange et al., 1998
Cyclothyrium sp.	Biphenyl, 2 mg	Sabouraud dextrose broth	4-Hydroxybiphenyl	96 h	da Silva et al., 2004

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Absidia pseudocylindrospora NRRL 2770 and *Absidia* sp. NRRL 1341 were the best performers. *Rhizopus japonicus* converted 4-chlorobiphenyl and 4,4′-dichlorobiphenyls (DCB) to hydroxylated metabolites (Wallnofer et al., 1973). The mechanism of hydroxylation of biphenyl by *Cunninghamella echinulata* was examined through two deuterated forms of substrates (Smith et al., 1981). The absence of isotope effect during the hydroxylation of biphenyl was noted. The presence of 1,2-hydride shift during 4-hydroxylation provided a link for the formation of an arene oxide as an intermediate. *Aspergillus parasiticus* resulted in the formation of 3-aryl muconolactones during the metabolism of biphenyl (Mobley et al., 1993). These products were formed only from biphenyls *para*-substituted at both phenyl rings and were not detected with unsubstituted biphenyls or *meta*-terphenyl.

5.7.2   Yeasts

Degradation of PCBs by yeasts is noted in Table 5.2. The oxidation of biphenyl occurs at the 4-position to form 4-hydroxybiphenyl by microsomal preparations from *Candida tropicalis* (Wiseman et al., 1975). Cerniglia et al. (1980) reported the oxidation of biphenyl to 4-hydroxyphenyl by *Oscillatoria* sp. strain JCM. Oxidation at the 4-position by fungi, yeast, and a cyanobacterium suggests the similarity found in the mammalian metabolism of biphenyl and chlorinated biphenyls. Glucose- or phenol-grown cells of *Trichosporon mucoides* were shown to have the ability to transform biphenyl into a large variety of mono-, di-, and trihydroxylated products, hydroxylated on one or both rings within 48 hours (Sietmann et al., 2001). Some of the metabolites accumulated in the culture supernatant as dead-end products and *ortho*-substituted dihydroxylated biphenyls were substrates for further oxidation and ring fission. In general, the transformation pathway of biphenyl was not affected by the mode of growth. The only exception was 2,3-dihydroxybiphenyl, in which transformation occurred only by phenol-grown cells. Phenyl derivatives of hydroxymuconic acids and the corresponding pyrones formed due to ring fission. Oxidation and ring fission of 3,4-dihydroxybiphenyl resulted in the formation of seven novel metabolites. The pathway for the biotransformation of biphenyl by *Trichosporon mucoides* is shown in Figure 5.1. All ring fission products are considerably less toxic than the hydroxylated derivatives.

Cells of *Debaryomyces vanrijiae* SBUG 770 grown with glucose were shown to have the ability to oxidize biphenyl to 4-hydroxybiphenyl as the main metabolite and 2-hydroxybiphenyl in minor amounts within 70 hours (Lange et al., 1998). When grown with tetradecane, cells of the yeast strain exhibited a lower ability to degrade biphenyl with the accumulation of small amounts of the hydroxylated metabolites. Under these conditions, 2-hydroxybiphenyl was slowly oxidized to 2,5-dihydroxybiphenyl, and 4-hydroxybiphenyl was rapidly metabolized, producing small amounts of two metabolites. One was identified as 3,4-dihydroxybiphenyl and
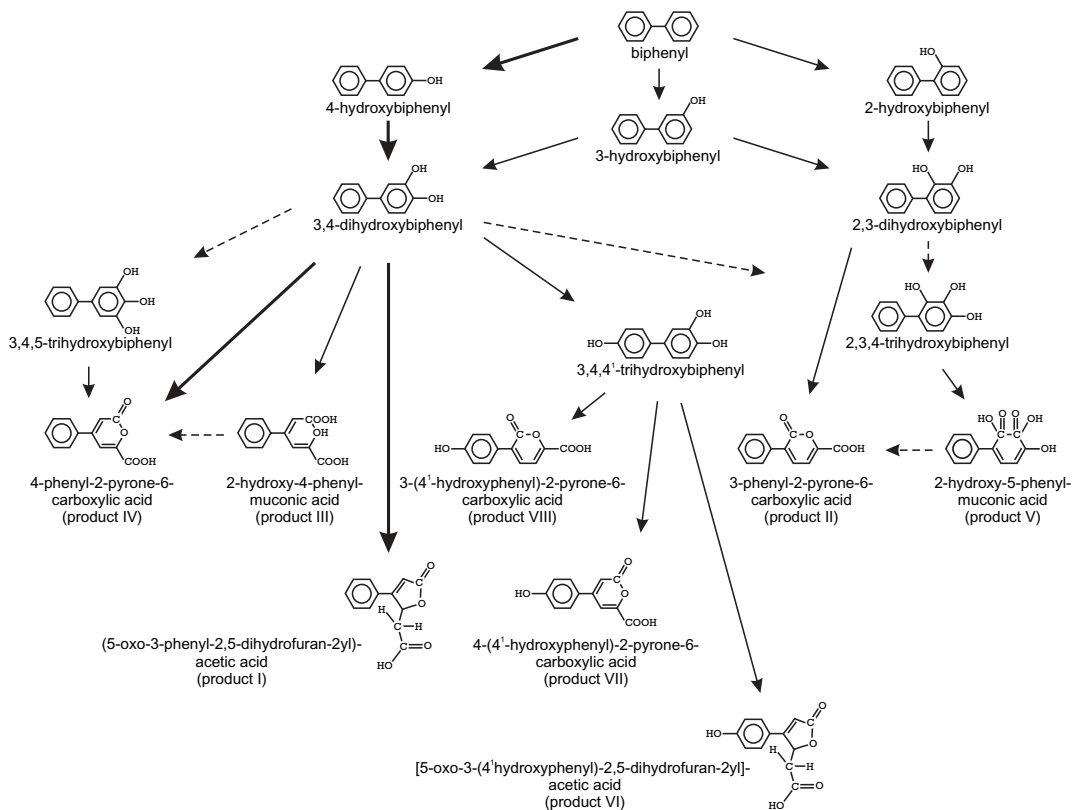
Figure 5.1 Tentative pathway for transformation of biphenyl by *Trichosporon mucoides* SBUG 801. [Reprinted from Sietmann et al. (2001), copyright © with permission from the American Society for Microbiology and the authors.]

second as 4-phenylmuconolactone. This lactone is also formed from 3, 4-dihydroxybiphenyl as a substrate. However, the oxidation of 4-hydroxybiphenyl produced the ring fission metabolite 4-phenyl-2-pyrone-6-carboxylic acid via 3,4-dihydroxybiphenyl. It appeared that ring cleavage of the aromatic structure took place after introduction of a third —OH group into the dihydroxylated intermediate.

Such a ring cleavage is also known during the transformation of diphenyl ether by *Trichosporon beigelii* SBUG 752 (Schauer et al., 1995). In addition, several oxidation products are formed during transformation. A monooxygenation step producing 2-, 3-, and 4-hydroxydiphenyl ethers (47:5:48) initiates this, and further oxidation results in 3,4-dihydroxydiphenyl ether. Further oxidation of 3- and 4-hydroxydiphenyl ethers produces 3,4-dihydroxydiphenyl ether and 6-carboxy-4-phenoxy-2-pyrone.

5.7.3   White-Rot Fungi

White-rot fungi have shown their ability to degrade many complex mixtures of PCBs and single congeners. Degradation of these xenobiotics is mediated by a lignin-degrading enzyme system of the fungus secreted in the extracellular environment. Major components of the lignin-degrading enzyme system include lignin peroxidases (LiPs), Mn(II)-dependent peroxidases (MnPs), and H_2O_2-producing system. These are induced during secondary metabolism under nutrient-limiting culture conditions. Many white-rot fungi have the ability to degrade a wide variety of PCBs. Besides *Phanerochaete chrysosporium*, several other white-rot fungi [e.g., *Pleurotus ostreatus* (Beaudette et al., 1998; Kubatova et al., 2001), *Coriolopsis polysona* (Vyas et al., 1994; Novotny et al., 1997), *Coriolus* (*Trametes*) *versicolor* (Beaudette et al., 1998; Cloete and Celliers, 1999; Koller et al., 2000). *Bjerkandera adusta* (Beaudette et al., 1998), *Lentinus* (*Lentinula*) *edodes* (Sasek et al., 1993; Ruiz-Aguilar et al., 2002)] are also known to metabolize PCBs.

5.7.3.1   White-Rot Fungal Bioreactors. White-rot fungi (e.g., *Pleurotus ostreatus* and *Trametes versicolor*) in a solid-state system (Zeddel et al., 1993) have been shown able to degrade PCBs. Three types of experiments were performed: (1) batch cultures were conducted with PCB-soaked wood chips for the degradation of PCBs; (2) closed 700-ml glass tubes 7 cm in diameter were used with air ventilation from the bottom to the top, and porous clay was filled at the bottom of the bioreactor to obtain a water sink to optimize the water tension in the hole of the tube; and (3) glass tubes were prepared in the same way as in type 2. After loading contaminants on the soil, a homogenization procedure was employed before mixing wood chips and fungal mycelia. Dried wood chips were added to the soil mud until it solidified into soil aggregates and was inoculated with *Pleurotus ostreatus* (3% w/w).

Phanerochaete chrysosporium, *Trametes versicolor*, and *Coriolopsis polysona* have been grown in malt extract medium with glucose and thus homogenized for the preparation of inoculum (Vyas et al., 1994). Fungi were grown in the presence of air before being spiked with [^{14}C]-3,3′,4,4′-tetrachlorobiphenyl. Oxygen was flushed immediately after spiking and then after 1 week. The $^{14}CO_2$ in headspace air was bubbled in 2 M ethanolamine as a $^{14}CO_2$ trap, and the radioactivity was measured. Specifically designed tube reactors were employed for the biodegradation of contaminated soil by *Pleurotus ostreatus* (Kubatova et al., 2001). The tube (3.5 cm in diameter and 24 cm in length) was divided into two compartments by a fine nylon net. In one compartment, the fungus was inoculated to the straw and mycelium grew through the net to the other compartment, which contained sterilized soil contaminated with a commercial mixture of Delor 103. The system was aerated and moistened during the course of a 2-month period. The reader is referred to Novotny et al. (1999) for a detailed description of this experimental procedure.

5.7.3.2 Degradation and Mineralization. Table 5.3 notes the degradation and mineralization of PCBs by white-rot fungi. Biphenyl and two model chlorinated biphenyls (i.e., 2-chlorobiphenyl and 2,2′,4,4′-tetrachlorobiphenyl) were degraded in suspended cultures by *Phanerochaete chrysosporium* (Thomas et al., 1992). Extensive mineralization of radiolabeled biphenyl (22.5%) and 2-chlorobiphenyl (15.6%) to CO_2 resulted within 30 days. No correlation existed between the rate of mineralization and the production of LiP or MnP. This suggests initial oxidation of PCBs by an unknown enzymatic system. Kurek and Odier (1990) reported the presence of LiP isozymes bound to mycelia. This may also be due to the cell wall–bound form of unreleased LiP in the culture. The higher chlorinated PCB, 2,2′,4,4′-tetrachlorobiphenyl, is degraded to CO_2 less readily and becomes associated with the mycelial biomass. *P. chrysosporium* degraded 75% of the congeners in Aroclor 1242 at a low initial concentration of 0.90 mg/l after 3 weeks (Asther et al., 1987).

Most of the congeners in Aroclor 1242 and 1254 are degraded extensively in both nutrient-limited (ligninolytic) and nutrient-rich (nonligninolytic) media by *P. chrysosporium* (Yadav et al., 1995). Extensive degradation occurred in congeners with a varying number of *ortho-*, *para-*, and *meta-*chlorines, suggesting relative nonspecificly for the position of chlorine substitutions on the biphenyl ring. This behavior is due to a free radical attack mechanism and is also known in the degradation of a wide variety of other aromatic compounds, such as chlorophenols and dioxins (Hammel, 1992). Malt extract medium supports maximum degradation (18.4% on a molar basis) of Aroclor 1260 as well as degradation of most of the congeners. The reductive dechlorination of certain components of Aroclor 1260 is known, but without noticeable reduction on a molar quantity (Quensen et al., 1990; GE, 1993). This was the first conclusive report on the degradation of PCBs in Aroclor 1260 by any microorganism.

Phanerochaete chrysosporium has the ability to transform three model PCBs, 4,4′-dichlorobiphenyl, 3,3′,4,4′-tetrachlorobiphenyl, and 2,2′,4,4′,5,5′-hexachlorobiphenyl, in liquid culture (Dietrich et al., 1995). This study contradicted the *para*-recalcitrance, as the mineralization of 2-chlorobiphenyl was more or less equivalent to that reported by Thomas et al. (1992). The ^{14}C partitioning dynamics to dichlorinated biphenyls exhibited the nonspecific adsorption of PCBs to the fungal hyphae. *P. chrysosporium* revealed higher mineralization rates of PCBs in cultures spiked with 30.14 nM (8.8 μg) 3,3′,4,4′-tetrachlorobiphenyl (PCB 77) than at 513.7 nM (150 μg) (Vyas et al., 1994). *C. polysona* mineralized PCB 77 poorly at higher concentrations but displayed similarity in the ^{14}C-radioactivity distribution pattern. Significant improvement in mineralization occurred in cultures spiked with lower concentrations. This correlates with the size of the intracellular pool of water-soluble radiolabel, which is lower at higher concentrations than at lower concentrations. *P. chrysosporium* increases intracellular degradation of PCB at low concentration and contributes significantly to the overall mineralization.

TABLE 5.3 Degradation and Mineralization of Polychlorinated Biphenyls by White-Rot Fungi

White-Rot Fungus	PCB Conc.	Medium	Degradation/ Mineralization Rate (%)	Duration (days)	Reference
Phanerochaete chrysosporium	Aroclor 1242, 10 mg/l Aroclor 1254, 10 mg/l Aroclor 1260, 10 mg/l	Low-N, high-N media and malt extract medium	60.9 30.5 17.6	30	Yadav et al., 1995
P. chrysosporium	Delor 103, 1 mg/l Delor 105, 1 mg/l	Nonlimited N media	55 58	9	Krcmar and Ulrich, 1998
P. chrysosporium *Trametes versicolor* *Coriolopsis polysona*	Delor 106, 0.9 mg/l	N-limited mineral media	25 50 41	21	Novotny et al., 1997
T. versicolor	PCB extract, 1800 mg/l	N-limited	70	10	Ruiz-Aguilar et al., 2002
P. chrysosporium	from contaminated soil, 600 mg/l 3000 mg/l	Mineral medium	73 34		
Lentinus (Lentinula) edodes	600 mg/l and 1800 mg/l		33		
Bjerkandera adusta UAMH 7308	2,3-Dichlorobiphenyl, 10 µg/ml 4,4′-Dichlorobiphenyl, 10 µg/ml 2,4′,5-Trichlorobiphenyl, 10 µg/ml 2,2′,4,4′-Tetrachlorobiphenyl, 10 µg/ml 2,2′,5,5′-Tetrachlorobiphenyl, 10 µg/ml 2,2′,4,4′,5,5′-Hexachlorobiphenyl, 10 µg/ml 2,4′,5-[U-^{14}C]Trichlorobiphenyl, 10 µg/ml	N-deficient medium	93 55 82 82 72 30 4.8 $^{14}CO_2$	21 22	Beaudette et al., 1998

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Species / strain	Compound	Medium	Value	Ref.
B. adusta UAMH 8258	2,4′,5-[U-^{14}C]Trichlorobiphenyl, 10μg/ml		6.9 $^{14}CO_2$	22
Pleurotus ostreatus UAMH 7972	2,3-Dichlorobiphenyl, 10μg/ml	N-deficient medium	88	21
	4,4′-Dichlorobiphenyl, 10μg/ml		28	
	2,4′,5-Trichlorobiphenyl, 10μg/ml		63	
	2,2′,4,4′-Tetrachlorobiphenyl, 10μg/ml		60	
	2,2′,5,5′-Tetrachlorobiphenyl, 10μg/ml		42	
	2,2′,4,4,5,5′-Hexachlorobiphenyl, 10μg/ml		12	
P. ostreatus UAMH 7964	2,4′,5-[U-^{14}C]Trichlorobiphenyl, 10μg/ml		3.9 $^{14}CO_2$	22
T. versicolor UAMH 8272	2,3-Dichlorobiphenyl, 10μg/ml	N-deficient medium	96	21
	4,4′-Dichlorobiphenyl, 10μg/ml		21	
	2,4′,5-Trichlorobiphenyl, 10μg/ml		43	
	2,2′,4,4′-Tetrachlorobiphenyl, 10μg/ml		12	
	2,2′,5,5′-Tetrachlorobiphenyl, 10μg/ml		50	
	2,2′,4,4,5,5′-Hexachlorobiphenyl, 10μg/ml		13	
	2,4′,5-[U-^{14}C]Trichlorobiphenyl, 10μg/ml		11 $^{14}CO_2$	22

Delor 103 or Delor 105 (as equivalents of Aroclor 1242 and Aroclor 1254) are not degraded under nitrogen-limited conditions but are reduced in non-limited nitrogen conditions by *P. chrysosporium* (Krcmar and Ulrich, 1998). LiP or MnP activity was not detected under nonlimited nitrogen conditions. Degradation of di- tri-, tetra-, penta-, hexa-, and hepta-congeners exhibited no significant differences. Both high and stable activities of LiP, MnP, Mn-independent peroxidase (MIP), and laccase are responsible for efficient degradation of PCBs by white-rot fungi (Novotny et al., 1997). *Trametes multicolor* reduced an overall 80% of the initial concentration of 2,5-dichlorobiphenyl (PCB 9) after 4 weeks (Koller et al., 2000). This is due to adsorption of PCB 9, and about 20% of PCB 9 can be extracted from the mycelium. This demonstrates the occurrence of degradation and adsorption/desorption simultaneously. Adsorption takes place due to reduction in the process of degradation. Nearly 40 components of the 41 major peaks of PCB 48 (includes di- to hexachlorobiphenyls) were degraded within 60 days by the edible mushroom Maitake, *Grifola frondosa* M51 (Seto et al., 1999).

White-rot fungi also have the ability to degrade PCBs extracted from historically contaminated soil (Ruiz-Aguilar et al., 2002). Tween 80 could emulsify the PCB extract and had no effect on fungal growth. Triton X-100 and Tergitol NP-10 inhibited fungal growth by 75 to 95%. *P. chrysosporium* was most effective for PCB degradation at an initial concentration of 3000 mg/l and removed up to 34% of the PCB mixture. *P. chrysosporium* and *L. edodes* accumulated low chlorinated congeners, and *T. versicolor* transformed both low and high chlorinated congeners of PCBs. This is the first report of PCB degradation at high concentration in a liquid culture by white-rot fungi.

Bjerkandera adusta UAMH 7308 and *B. adusta* UAMH 8258 were shown to be the most active degraders of PCB congeners (Beaudette et al., 1998). This is the first report of degradation of PCB congeners by species of *Bjerkandera*. *Trametes versicolor* degraded 2,3-dichlorobiphenyl extensively (96%), but was less effective with other congeners. The congener loss ranged from 40 to 96%, except *P. chrysosporium*. White-rot fungi showed mineralization of 2,4′,5-[U-^{14}C]trichlorobiphenyl to ^{14}CO$_2$. Low levels of LiP and MnP activities were detected in these fungi. However, regression analysis of biodegradation, mineralization, and LiP and MnP activities of all 14 strains revealed no linear correlation. Recently, Beaudette et al. (2000) demonstrated enhanced mineralization of a PCB congener by the application of a low concentration of surfactant. However, the surfactant did not increase the overall metabolism.

5.7.3.3 *Effects of Chlorination Grades and Patterns.* In general, most of the studies presented so far confirm the decrease in level of mineralization with an increase in the degree of PCB chlorination (Zeddel et al., 1993; Yadav et al., 1995; Beaudette et al., 1998; Kubatova et al., 2001). Thomas et al. (1992) determined the decrease in levels of mineralization in the following order: [^{14}C]biphenyl (23%) > 2-[^{14}C]chlorobiphenyl (16%) > 2,2′,4,4′-

[^{14}C]tetrachlorobiphenyl (TCB) (10%). The data also show the effect of the congener chlorination pattern to PCB degradation by *P. chrysosporium*. About 10% of 2,2′,4,4′-[^{14}C]TCB in 32 days and <1% of 3,3′,4,4′-[^{14}C]TCB were mineralized in 30 days (Bumpus et al., 1985; Thomas et al., 1992). The PCB chlorination pattern also affected the degradation of technical PCB mixtures by *P. chrysosporium*. Mineralization of 20% of [^{14}C]Aroclor 1242 (42% chlorine by weight) (Bumpus et al., 1985) and 10 to 14% of [^{14}C]Aroclor 1254 (54% chlorine by weight) (Bumpus et al., 1985; Eaton, 1985) by *P. chrysosporium* has been determined. Zeddel et al. (1993) reported the degradation of nonspecific PCB mixture by *P. chrysosporium* to mono- and dichlorinated congeners.

5.7.3.4   Metabolic Products and Pathways. Metabolic products produced during PCB degradation by white-rot fungi are listed in Table 5.4. Dietrich et al. (1995) produced the first conclusive report describing the metabolic products during PCB degradation by *P. chrysosporium*. These and prior studies suggest the characteristic chlorobenzoates as metabolic intermediates in PCB degradation pathways in fungi. Chlorobenzoates appear to be common metabolic products in bacterial and fungal PCB degradation pathways, but the mechanism of their formation may or may not be the same. Dioxygenases are responsible for the *meta*-pathway, catalyzing the 2,3-dihydroxylation and extradiol ring fission. Three possibilities have been proposed for the relation of 4-chlorobenzyl alcohol (4-CBAlc) to 4-chlorobenzoic acid (4-CBA) in the fungal PCB degradation pathway: (1) transformation of a PCB fission-ring product to 4-CBA and then reduction to 4-CBAlc, (2) formation of 4-CBAlc and subsequent oxidation to 4-CBA, and (3) independent production of both 4-CBA and 4-CBAlc by parallel metabolic pathways. In *P. chrysosporium*, two mechanisms appear to be responsible for the metabolism of a wide variety of diphenyl compounds (Hiratsuka et al., 2005): the LiP-catalyzed one-electron oxidation of the aromatic ring producing the aryl cation radical, and the hydroxylation reactions of the aromatic ring by cytochrome P450 enzymes.

Glucose-grown cells of *Pycnosporus cinnabarinus* have the ability to hydroxylate biphenyl and diphenyl ether in a Sabouraud medium culture (Jonas et al., 2000). Only low levels of hydroxylated metabolites equivalent to 1% of the parent compound were produced after 3 days. Laccase is the only ligninolytic enzyme found in the culture supernatant. All three strains of *Trametes versicolor* produce the same pattern of metabolites on diphenyl ether and its halogenated derivatives, 4-bromo- and 4-chlorodiphenyl ethers (Hundt et al., 1999). No metabolites are produced by cells of *T. versicolor* from diphenyl ethers during incubation with 1-aminobenzotriazole, a cytochrome P450 inhibitor. Cell-free supernatants of whole cultures showing MnP and laccase activities are unable to transform any of the diphenyl ethers. The pathway proposed for the degradation of diphenyl ethers by *T. versicolor* is shown in Figure 5.2.

TABLE 5.4 Metabolic Products During Degradation of Polychlorinated Biphenyls by White-Rot Fungi

White-Rot Fungus	PCB Conc.	Medium	Degradation/ Mineralization Rate (%)	Metabolic Products	Duration (days)	Reference
Phanerochaete chrysosporium	4,4'-Dichlorobiphenyl, 0.68 μg/g	Liquid culture	11.65 $^{14}CO_2$	4-Chlorobenzoic acid, 4-chlorobenzyl alcohol	28	Dietrich et al., 1995
	3,3',4,4'-Tetrachlorobiphenyl, 1.07 μg/g		Negligible			
	2,2',4,4',5,5'-Hexachlorobiphenyl, 1 μg/g		Negligible			
Pycnosporus cinnabarinus	Biphenyl, 480 μM Diphenyl ether, 480 μM	Sabouraud medium		2- and 4-Hydroxybiphenyls 2- and 4-Hydroxydiphenyl ethers	3	Jonas et al., 2000
Trametes versicolor	Diphenyl ether	N-rich medium		2- and 4-Hydroxydiphenyl ethers, 2-hydroxy-4-phenoxymuconic acid, 6-carboxy-4-phenoxy-2-pyrone	7	Hundt et al., 1999
	4-Bromo- and 4-chlorodiphenyl ether			Brominated and chlorinated derivatives of the products above and 4-bromo- and 4-chlorophenol		

Organism	Substrate	Culture	Conversion	Products		Reference
Trametes multicolor	PCB 9 (2,5-dichlorobiphenyl), 5µM	Nutrient broth	80	Chlorobenzenes, chlorophenols, alkylated benzenes	42	Koller et al., 2000
P. chrysosporium	4,4′-Dichlorobiphenyl	Culture		4-Chlorobenzoate, 4-chlorobenzyl alcohol		Hickey, 1996
Grifola frondosa	PCB 48, 2,4′,2,3, 10µg/ml; PCB 48, 2,6,3/2,3,6, 10µg/ml; 4-Chlorobiphenyl, 25µg/ml	Low-N medium	100; 100	Dichloromethoxyphenol; Three isomers of hydroxy-4-chlorobiphenyl	30	Seto et al., 1999
P. chrysosporium	3,3′,4,4′-Tetrachlorobiphenyl, 8.8µg	N-limited stationary cultures	1.39 $^{14}CO_2$	Water-soluble products	28	Vyas et al., 1994
T. versicolor *Coriolopsis polysona*			0.40 $^{14}CO_2$; 0.015 $^{14}CO_2$			
P. chrysosporium	Biphenyl, 2.26µM; 2-Chlorobiphenyl, 95nM; 2,2′,4,4′-Tetrachlorobiphenyl, 40nM	Suspended cultures	22.5 $^{14}CO_2$; 15.6 $^{14}CO_2$; 9.6 $^{14}CO_2$	Water-soluble products	30	Thomas et al., 1992
P. chrysosporium	Aroclor 1254, 250µg/l	W medium	7.1 $^{14}CO_2$	Water-soluble products	31	Eaton, 1985

2-hydroxydiphenyl ether

3-hydroxydiphenyl ether

4-hydroxydiphenyl ether

trihydroxylated intermediate

2-hydroxy-4-phenoxymuconic acid

5-carboxy-4-phenoxy-2-pyrone

X = H, Br, Cl
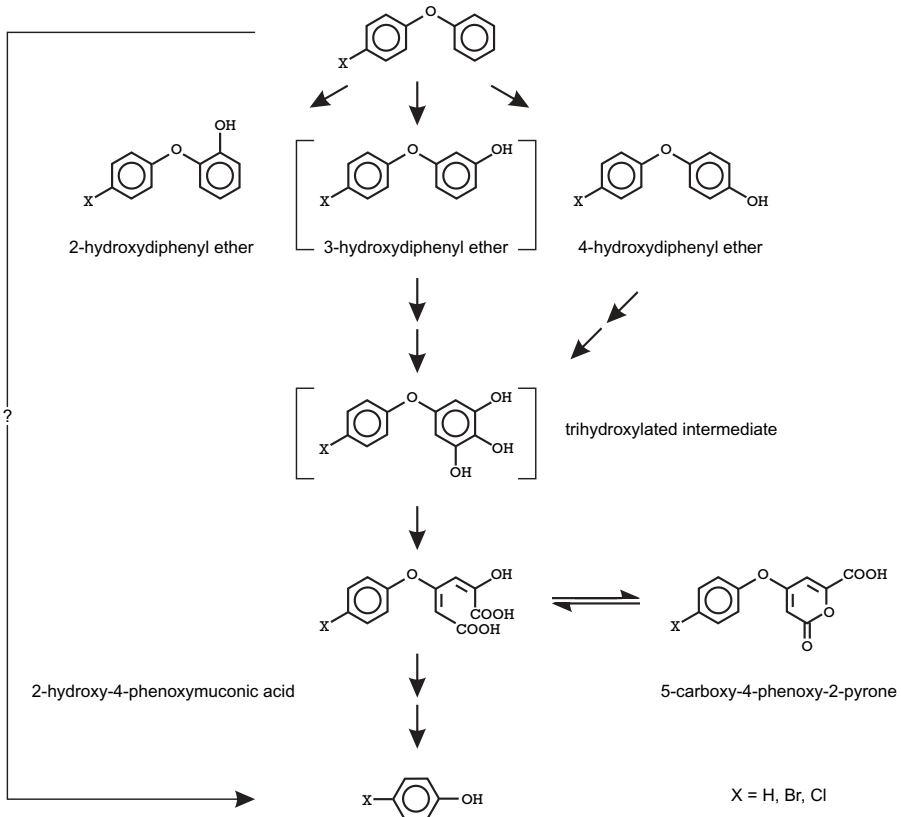
Figure 5.2 Tentative pathway for degradation of diphenyl ethers by *Trametes versicolor*. [Reprinted from Hundt et al. (1999), copyright © with permission from Springer Science and Business Media and the authors.]

Trametes multicolor produces a wide variety of metabolic products due to degradation of 2,5-dichlorobiphenyl (PCB 9) (Koller et al., 2000). The compounds identified vary from oxidized products such as chlorinated phenols and carboxylated benzenes to reduced metabolites as chlorinated benzenes. The formation of several metabolites suggests complex reaction pathways. In vitro experiments show the mineralization of 4,4′-dichlorobiphenyl using *P. chrysosporium* with the formation of 4-chlorobenzoate and 4-chlorobenzyl alcohol as its metabolites (Hickey, 1996). *Grifola frondosa* M51 accumulates dichloromethoxyphenol during degradation of PCB 48 in low-nitrogen medium (Seto et al., 1999). This metabolite appears to be produced as a result of a ring cleavage of PCB degradation. The fungus accumulates three metabolites during degradation of 4-chlorobiphenyl and seem to be the isomers of hydroxy-4-chlorobiphenyl.

5.7.3.5 *Role of Manganese in PCB Degradation.* Addition of Mn(IV) oxide in cultures was shown to improve the production, enzymatic activity, and stability of LiP of *P. chrysosporium* (Kern, 1989). The chelating agents stimulated MnP in the presence of Mn(II). Addition of solid manganese (IV) oxide improved the production of MnP and H_2O_2 and enhanced the rate of degradation of Aroclor 1254 in an N-limited medium by *Coriolus versicolor* (Cloete and Celliers, 1999). H_2O_2 production was enhanced by a 34.5 mM concentration of MnO_2. Aroclor 1242 was also removed in the absence of MnO_2, suggesting the presence of LiP or nonspecific absorption. About 84% of Aroclor was eliminated in cultures containing 57.5 mM MnO_2 in 6 days. Penta- and hexachlorobiphenyls were removed at a slower rate than tri- and tetrachlorobiphenyl in cultures with MnP or LiP.

5.7.3.6 *PCB Bioremediation in Soils.* Colvin and Nelson (1990) presented a field demonstration and described the advantages of a process using *P. chrysosporium.* The U.S. Environmental Protection Agency (EPA) issued the first research and development permit for the bioremediation of PCBs using white-rot fungi. Degradation of Aroclors occurs and the less chlorinated congeners decrease on a percentage basis. However, growth does not occur at temperatures below 15°C. Table 5.5 lists the bioremediation of PCBs in soils by white-rot fungi.

Similar rates of degradation for mono-, di-, and trichlorinated PCBs in different media revealed certain similarities in the activities of fungi (Zeddel et al., 1993). Based on the substitution pattern, up to 50% of penta- and hexachlorobiphenyls was degraded. The lower chlorinated 4,4′-dichlorobiphenyl was not degraded. The enzymatic system preferred the substituted *para*-position. Low reactivity of *ortho*-position may result in slower degradation of *para*-isomers. Slow degradation of PCBs occurs when adsorbed on a soil surface. Growth and degradation of PCBs are not achieved by the addition of lignosulfonate, an inducer of ligninolytic activity. Bedard (1990) demonstrated about 50% degradation in the upper few centimeters of PCB-contaminated soil and 10% in the deeper layers due to the use of bacterial cultures.

A model system has been established at the laboratory scale for the transformation of PCB-contaminated soil through bioaugmentation using *P. chrysosporium* grown on sugarcane bagasse pith (Fernandez-Sanchez et al., 1999). Nearly 90% degradation occurred with a young inoculum of mycelia. This treatment appears to be suitable in a remediation process, due to the shorter time necessary to produce inoculum along with the high level of degradation. In general, the most chlorinated congeners dehalogenate to the least chlorinated congeners. The main transformation occurs in pentachlorobiphenyls. Viney and Bewley (1990) showed a 50% reduction in the pentachlorinated biphenyl and a 53% reduction in the hexachlorinated biphenyl in *P. chrysosporium*–inoculated sand cultures. Of six strains, four

TABLE 5.5 Bioremediation of Polychlorinated Biphenyls in Soils by White-Rot Fungi

White-Rot Fungus	PCB/Conc.	Degradation/Mineralization Rate (%)	Duration (days)	Reference
Pleurotus ostreatus (four strains)	Delor 103, 1.021 mg/ml	40	60	Kubatova et al., 2001
P. ostreatus	PCB congener 11, 70μl PCB congener 77	$0.4\ CO_2$ $0.1\ CO_2$	60	Kubatova et al., 1998
Phanerochaete chrysosporium	PCB-contaminated soil (similar to Aroclor 1260)	90	60	Fernandez- Sanchez et al., 1999
P. chrysosporium and four other white-rot fungi	Chlorinated mixture of PCBs (tetra- to hexachlorobiphenyls)	<6	40	Hickey, 1996
P. ostreatus	PCB mixture, 2500–3000mg/kg	95	35	Zeddel et al., 1993
Trametes versicolor	Pentachlorobiphenyl	50		
	Hexachlorobiphenyl loaded on wood chips	50		
P. ostreatus	Delor 106, 2.61 mg/kg	29	42	Sasek et al., 1993
Lentinus (*Lentinula*) *edodes*		24		
P. chrysosporium		11		

strains of *Pleurotus ostreatus* have the ability to eliminate 40% of Delor 103 in 2 months in a soil system (Kubatova et al., 2001). Only 0.47 and less than 0.1% of the PCB 11 and PCB 77 are mineralized in soil by *P. ostreatus*, respectively (Kubatova et al., 1998). *P. ostreatus*, *Lentinus* (*Lentinula*) *edodes*, and *P. chrysosporium* degraded nearly 29%, 24%, and 11% of Delor 106, respectively, after 6 weeks in pasteurized soil (Sasek et al., 1993). However, 50% Delor degraded in nonpasteurized soil by a mixture of white-rot fungi, yeasts, and bacteria after 11 weeks. *P. chrysosporium* and four other white-rot fungi produced extensive mycelial networks when inoculated into soil contaminated with a highly chlorinated mixture of PCBs (Hickey, 1996). However, only about a ≤6% reduction in certain congeners was found after 40 days. The white-rot fungi are the most effective to degrade the more lightly chlorinated PCBs, thus exhibiting similarities to aerobic bacteria.

5.7.3.7   Biotransformation of PCBs by Laccases. Laccases are versatile enzymes and occur widely in fungi. Their role in fungi is varied and involved in the regulation of morphology, the control of virulence and nutrition, and to delignify woody tissues. These are also useful for the degradation of a variety of persistent organic pollutants. Despite extensive studies, knowledge of the dehalogenation of PCBs by fungal laccases is fragmentary. Table 5.6 contains some studies on the degradation of PCBs by white-rot fungal laccases.

Chlorinated hydroxybiphenyls are transformed to oligomerization products by laccase from *Pycnosporus cinnabarinus* (Schultz et al., 2001). Transformation of 2-hydroxy-5-chlorobiphenyl by laccase results in one main product in the form of dimer, 5,5′-di-(2-hydroxybiphenyl). One coupling of 2-hydroxy-5-chlorobiphenyl occurs at position C5 in the *para*-position with respect to the —OH group, and thus both chlorine atoms are split off. Further products represent C—O and C—C linked dimers with no dechlorination. Transformation of 3-chloro-4-hydroxybiphenyl produces white precipitates and the formation C—O and C—C linked dimers with a different number of chlorines.

Transformation of 4-hydroxy-4′-chlorobiphenyl produced slightly violet precipitates and the formation C—C dimers and one or two chlorine atoms. Addition of 2-hydroxydiphenyl ether and 2-hydroxybiphenyl to cell-free supernatants containing laccase of *P. cinnabarinus* resulted in the formation of different-colored precipitates (Jonas et al., 2000). Transformation of 2-hydroxydiphenyl ether formed a red-brown precipitate, and that of 2-hydroxybiphenyl a red precipitate. Only 3% of the compounds added initially were detectable after 30 minutes of incubation with the culture filtrate. GC-MS analysis of the polymer mixture revealed pools of dimers and trimers with different binding types. Dimers with C—C bonds in the *para*-position to the hydroxyl group of the monomers have been identified as the major products.

TABLE 5.6 Biotransformation of Polychlorinated Biphenyls by White-Rot Fungal Laccases

White-Rot Fungus/Laccase Activity	PCB Conc.	Bioreactor	Biotransformation (%)	Time (hours/minutes)	Metabolic Products	Time (minutes)	Reference
Pycnosporus cinnabarinus/ 450 nM/ml per minute	2-Hydroxy-5-chlorobiphenyl, 2 mM	20-ml reaction tubes	92	24h	5,5'-Di-(2-hydroxybiphenyl), C—O and C—C linked dimers with no dechlorination	30m	Schultz et al., 2001
	3-Chloro-4-hydroxybiphenyl, 2 mM		97	24h	White precipitates, chlorinated C—O and C—C linked dimers	30m	
	4-Hydroxy-4'-chlorobiphenyl, 2 mM		89	24h	Slightly violet precipitates, chlorinated C—C linked dimers	30m	
P. cinnabarinus/ 200 nM/ml per minute	2-Hydroxybiphenyl, 215 μM		97	30m	Red precipitates, dimers and trimers with different binding types, 5,5-di-(2-hydroxybiphenyl)	5m	Jonas et al., 2000
	2-Hydroxydiphenyl ether, 215 μM		97	30m	Red-brown precipitates, dimers and trimers with different binding types, 5,5-di-(2-hydroxydiphenyl ether)	5m	

5.7.3.8 *Comparison with Bacterial Systems.* During the past decade, white-rot fungi became a model of research related to the degradation of various organic pollutants throughout the world. The knowledge produced as a result makes them unique and innovative organisms compared to bacteria that enjoyed well-established methods of bioremediation. The major differences include the possession of unusual and remarkable mechanisms that have great advantages over the bacterial systems. Understanding of the complexity of mechanisms will lead to the development of emerging applications in this method of bioremediation.

In bacterial systems, bacteria must be exposed to the compound, and the organic compounds should be available in adequate quantities so that sufficient synthesis of enzymes will take place for its degradation. On the contrary, white-rot fungi can be cultivated under nutrient-limited conditions, and the process of degradation is initiated. Such limiting conditions result in the induction of various degradative enzymes. The major advantage lies in the fact that the production of a lignin-degrading system does not depend on the compound. In addition, preconditioning to a specific compound is not required.

White-rot fungi have been shown to cultivate easily on cheap and waste nutrient sources such as wood chips, sawdust, and agricultural wastes, and to become established strongly in the field among the indigenous microorganisms. Bacteria do not utilize such nutrient sources as easily. The limited knowledge indicates the growth of white-rot fungi in different geographical areas, utilizing these sources of nutrients. These fungi have a strong ability to antagonize microbial growth during field trials. These fungi are known to produce hydroxyl free radicals that can result in the death of other microorganisms by oxidizing proteins and DNA. In addition, the fungus has the ability to adjust the pH of the surrounding environment, thus unbalancing the optimum pH of other microbes.

Another major difference of degradation lies in the order of kinetics. In bacterial systems, the metabolism is accompanied by enzymatic conversions and governed by kinetics of Michaelis–Menten order. Also, bacterial degradation depends on the solubility of compound or mixture of compounds. On the contrary, a free radical process and degradation of compounds by these fungi display pseudo-first-order kinetics. Several studies establish that the rate of disappearance or mineralization of compounds depends on its initial concentration. This reflects the capability of these fungi to achieve decontamination at the sites. Also, these fungi have the ability to convert insoluble organic complex mixtures by using a nonspecific free radical mechanism.

One of the most important advantages of white-rot fungi is the extracellular degradation of organic compounds. In other words, degradation of organic compounds does not require metabolism. The nonspecific nature of the mechanism of white-rot fungi can be a successful remedy for PCB contamination.

5.8   FUNGAL DEGRADATION OF DIOXINS

Dioxins and furans are some of the most toxic compounds known to science. Dioxins are released in the environment as the by-products of several industrial processes involving chlorine, such as waste incineration, chemical and pesticide manufacturing, and paper and pulp bleaching. These compounds are also found as impurities in pentachlorophenol, which is used as a wood preservative. These compounds are highly stable and lipophilic in nature.

Fungi are the most effective degraders of dioxins, and core studies have been performed by some researchers. Several strains of filamentous fungi have the ability to hydroxylate dibenzofuran, indicating a monooxygenase system (Hammer and Schauer, 1997). Eight strains of fungi metabolized dibenzofuran only to intermediates with hydrophilic characteristics. Six major metabolites were detected during the transformation of dibenzofuran by glucose-pregrown cells of *Trichosporon mucoides* (Hammer et al., 1998). Incubation with monohydroxylated dibenzofurans produces 2,3-dihydroxydibenzofuran and its ring cleavage product 2-(1-carboxy methylidene)-2,3-dihydrobenzo[*b*]furanylidene glycolic acid. Biotransformation of dibenzofuran and related derivatives by the Imperfect soil fungus *Paecilomyces lilacinus* proceeded with phase I and phase II reactions to produce 14 hydroxylated and excretable sugar conjugative metabolites (Gesell et al., 2004). Biotransformation was catalyzed by two separate hydroxylation steps, and 4-mono- and 4-dihydroxylated dibenzofurans accumulated. Both aromatic rings were hydroxylated to form 2,7-, 3,7-, and 2,8-dihydroxydibenzofurans, and further oxidation resulted in ring cleavage of dibenzofuran. The ring-fission products include benzo[*b*]furo[3,2-*d*]-2-pyrone-6-carboxylic acid and [2-(1-carboxy-methylidene)benzofuran-3-ylidene]hydroxyacetic acid, and its derivatives are hydroxylated at C7 and C8 at the noncleaved ring. Riboside conjugates of 2- and 3-hydroxydibenzofurans are among other metabolites.

A cyclic ether degrading fungus, *Cordyceps sinensis* strain A, shows a new degradation pathway for dioxins (Nakamiya et al., 2005). Degradation of dibenzo-*p*-dioxin (DD) produces catechol, which is subsequently metabolized to *cis,cis*-muconates. Catechol, mono- and dichloro-catechol, and *cis,cis*-muconates are formed as a result of the metabolism of 2,3,7-trichlorodibenzo-*p*-dioxin (2,3,7-triCDD). Degradation of octachlorodibenzo-*p*-dioxin (octaCDD) yields to catechol, mono- and trichloro-catechol, and *cis,cis*-muconates. Dechlorination appears to occur in the degradation of octaCDD to catechols and in the subsequent degradation of catechols and/or *cis,cis*-muconates.

A white-rot fungus, *Phlebia lindtneri*, has been shown to biotransform model polychlorinated dibenzo-*p*-dioxins (PCDDs), such as 2,7-dichloro-, 2,3,7-trichloro-, and 1,2,6,7- and 1,2,8,9-tetrachlorodibenzo-*p*-dioxins to hydroxylated and methoxylated compounds (Kamei and Kondo, 2005). The degradation rate of 1,2,6,7-tetrachlorodibenzo-*p*-dioxin is higher than that of

2,3,7-trichlorodibenzo-*p*-dioxin. The degradation of the PCDDs is related to the chlorination patterns of these substrates. *P. lindtneri* also metabolized 2,8-dichlorodibenzofuran (2,8-diCDF) to hydroxy-diCDF (Mori and Kondo, 2002). The recombinant yeast cells expressing rat CYP1A1 and CYP1A2 each convert most of 2,3,7-trichlorodibenzo-*p*-dioxin to 8-hydroxy-2,3,7-trichlorodibenzo-*p*-dioxin and subsequently to more hydrophilic compounds with cleavage of ethereal bridges (Sakaki et al., 2002). The metabolism showed three types of hydroxylations and opening of the dioxin ring. These findings suggest the possibility of use of yeast-expressing mammalian cytochrome P450 in the bioremediation of dioxin-contaminated soils. Three transformants of *Coriolus hirsutus* efficiently converted 2,7/2,8-dichlorodibenzo-*p*-dioxins (2,7/2,8-DCDDs) through the rat cytochrome P450, CYP1A1 enzyme (Orihara et al., 2005).

  Phanerochaete sordida YK-264 degrades nearly 60% of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (tetraCDD) (Takada et al., 1996). Valli et al. (1992) suggested a multistep pathway for the degradation of 2,7-dichlorodibenzo-*p*-dioxin (2,7-diCDD) by LiP and MnP of *Phanerochaete chrysosporium*. In the first step, LiP oxidatively cleaves 2,7-diCDD to 4-chloro-1,2-benzoquinone, 2-hydroxy-1,4-benzoquinone, and chloride. Reduction of 4-chloro-1,2-benzoquinone yields to 1-chloro-3,4-dihydroxybenzene, which methylates to 1-chloro-3,4-dimethoxybenzene, and this subsequently oxidizes to 2-methoxy-1,4-benzoquinone and chloride. LiP reduces the metabolite 2-methoxy-1,4-benzoquinone to 2-methoxy-1,4-dihydroxybenzene, which is further oxidized to 4-hydroxy-1,2-benzoquinone, and this is subsequently reduced to yield 1,2,4-trihydroxybenzene. Reduction and ring cleavage of 1,2,4-trihydroxybenzene yield β-ketoadipic acid.

5.9   GENETIC MANIPULATION

Recent advances in biotechnology can open the door for the development of genes responsible for the mineralization of PCBs by fungi. Genes encoding LiP in 30 fungal species have been screened (Varela et al., 2000) that may open new frontiers for the degradation of PCBs. A dendogram illustrating a sequence relationship among 32 fungal peroxidases has been presented (Martinez, 2002). A great future lies in successful genetic splicing and bringing together pathway fragments with a view to constructing an entirely new white-rot fungus that can utilize PCBs as the sole source of carbon.

5.10   CONCLUSIONS AND FUTURE PERSPECTIVES

Virtually, no work is known on the screening of fungal species for the degradation of PCBs. At present, there are not many PCB-degrading yeasts and fungi, so screening is important for the discovery of new fungal species. Most

studies conducted to date have used liquid culture media and controlled conditions in the laboratory. Some examples are known of the mineralization of PCBs, but the metabolic products have been identified in only a few cases. Despite 95% degradation of chlorinated biphenyls by *Trametes versicolor* (Zeddel et al., 1993), the mineralization rate obtained by white-rot fungi does not exceed 11% (Vyas et al., 1994; Dietrich et al., 1995; Beaudette et al., 1998). It seems that the metabolites formed due to degradation are difficult to analyze. Unfortunately, not much has been accomplished to date on the elucidation of metabolic pathways of any PCB by fungi. It is recommended that experiments be designed such that the maximum mineralization of PCBs can be deciphered by fungi. Inoculating contaminated soils with effective PCB degraders along with optimizing requirements can provide efficient mineralization. A combination of surfactant and yeasts or fungi, or yeast or fungi secreting surfactant, can also be tested to overcome the problems of bioavailability and degradation of PCBs.

In recent years, mechanisms of action of white-rot fungi related to organic pollutants have been understood that offer great potential for the use of PCB degradation. The contribution of the highly oxidation potential of a non-specific ligninolytic enzymatic system can be extended in the removal of complex mixtures of PCBs. The breakdown of readily available and readily utilizable materials such as wood chips is more economical than the cometabolism of PCBs by aerobic bacteria. White-rot fungi can easily compete with other microbes and be established in the surrounding environment. The process controls need to be optimized to take full advantage of the unique system. The projections indicate a more cost-effective type of bioremediation system than the current competitive technologies. The time is ripe to implement laboratory knowledge to design an applicable strategy for large-scale commercially viable white-rot bioremediation technology.

REFERENCES

Alexander, M. (1997) Sequestration and bioavailability of organic compounds in soil. In: *Environmentally Acceptable Endpoints in Soil*: *Risk-Based Approach to Contaminated Site Management Based on Availability of Chemicals in Soil*, D.G. Linz and D.V. Nakles, eds. American Academy of Environmental Engineers, Annapolis, MD, pp. 43–136.

Asther, M., G. Corrieu, R. Drapron, and E. Odier (1987) Effect of Tween 80 and oleic acid on ligninase production by *Phanerochaete chrysosporium* INA-12. *Enzyme Microb. Technol.* **9**: 245–249.

Baveye, P., and R. Bladon (1999) Bioavailability of organic xenobiotics in the environment: a critical perspective. In: *Bioavailability of Organic Xenobiotics in the Environment*, P. Baveye. J.-C. Block, and V.V. Goncharuk, eds. Kluwer, Boston, pp. 227–248.

Beaudette, L.A., S. Davies, P.M. Fedorak, O.P. Ward, and M.A. Pickard (1998) Comparison of gas chromatography and mineralization experiments for measuring loss

of selected polychlorinated biphenyl congeners in cultures of white rot fungi. *Appl. Environ. Microbiol.* **64**: 2020–2025.

Beaudette, L.A., O.P. Ward, M.A. Pickard, and P.M. Fedorak (2000) Low surfactant concentration increases fungal mineralization of a polychlorinated biphenyl congener but has no effect on overall metabolism. *Lett. Appl. Microbiol.* **30**: 155–160.

Bedard, D.L. (1990) Bacterial transformation of PCBs. In: *Biotechnology and Bio-degradation*, D. Kamely, A. Chakrabarty, and G.S. Omenn, eds. Gulf Publishing Co., Houston, TX, pp. 369–388.

Bedard, D.L., and J.F. Quensen III (1995) Microbial reductive dechlorination of polychlorinated biphenyls. In: *Microbial Transformation and Degradation of Toxic Organic Chemicals*, L.Y. Young and C.E. Cerniglia, eds. Wiley-Liss, New York, pp. 127–216.

Biggs, D.C., C.D. Powers, R.G. Rowland, H.B. O'Conners, and C.F. Wurster (1980) Uptake of polychlorinated biphenyls by natural phytoplankton assemblages: field and laboratory determination of ^{14}C-PCB particle-water index of sorption. *Environ. Pollut.* **22**: 101–110.

Bosma, T.N.P., P.J.M. Middeldorp, G. Schraa, and A.J.B. Zehnder (1997) Mass trans-fer limitation of biotransformation: quantifying bioavailability. *Environ. Sci. Technol.* **31**: 248–252.

Boyle, A.W., C.J. Silvin, J.P. Hassett, J.P. Nakas, and S.W. Tanenbaum (1992) Bacte-rial PCB biodegradation. *Biodegradation* **3**: 285–298.

Bumpus, J.A., M. Tien, D. Wright, and S.D. Aust (1985) Oxidation of persistent environmental pollutants by a white rot fungus. *Science* **228**: 1434–1436.

Cerniglia, C.E., C. van Baalen, and D.T. Gibson (1980) Oxidation of biphenyl by the cyanobacterium, *Oscillatoria* sp. strain JCM. *Arch. Microbiol.* **125**: 203–207.

Cloete, T.E., and L. Celliers (1999) Removal of Aroclor 1254 by the white rot fungus *Coriolus versicolor* in the presence of different concentrations of Mn(IV) oxide. *Int. Biodeterior. Biodegrad.* **44**: 243–253.

Cole, M.A., P.B. Reichart, and D.K. Button (1979) Reversible bioconcentration of monochlorobiphenyls by *Rhodotorula rubra*: correlations with aqueous solubility of substrate. *Bull. Environ. Contam. Toxicol.* **23**: 44–50.

Colvin, S., and D. Nelson (1990) White rot fungi in biodegradation. In: *Proceedings of Hazardous Waste Management in the 90's: Moving from Remediation to Practi-cal Preventive Strategies*. A&WMA, Pittsburgh, PA, pp. 141–146.

da Silva, M., E. Esposito, J.D. Moody, V.P. Canhos, and C.E. Cerniglia (2004) Metab-olism of aromatic hydrocarbons by the filamentous fungus *Cyclothyrium* sp. *Che-mosphere* **57**: 943–952.

Dietrich, D., W.J. Hickey, and R. Lamar (1995) Degradation of 4,4′-dichlorobiphenyl, 3,3′,4,4′-tetrachlorobiphenyl, and 2,2′,3,3′,4,4′-hexachlorobiphenyl by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **61**: 3904–3909.

Dmochewitz, S., and K. Ballschmiter (1988) Microbial transformation of technical mixtures of polychlorinated biphenyls (PCB) by the fungus *Aspergillus niger*. *Chemosphere* **17**: 111–121.

Dodge, R.H., C.E. Cerniglia, and D.T. Gibson (1979) Fungal metabolism of biphenyl. *Biochem. J.* **178**: 223–230.

Eaton, D.C. (1985) Mineralization of polychlorinated biphenyls by *Phanerochaete chrysosporium*: a ligninolytic fungus. *Enzyme Microb. Technol.* **7**: 194–196.

Erickson, M.D. (1997) *Analytical Chemistry of PCBs*. Lewis Publishers, Chelsea, MI.

Fernandez-Sanchez, J.M., G. Ruiz-Aguilar, and R. Rodriguez-Vazquez (1999) Polychlorinated biphenyls transformation by bioaugmentation in solid culture. In: *Proceedings of the Fifth International In Situ and On-Site Bioremediation Symposium*, B.C. Alleman and A. Leeson, eds. Battelle Press, Columbus, OH, Vol. 7, pp. 173–178.

GE (1993) General Electric Company Research and Development Program for the Destruction of PCBs: Twelfth Progress Report for the Period Aug. 1, 1992–July 31, 1993. GE Corporate Research and Development, Schenectady, NY.

Gesell, M., E. Hammer, A. Mikolasch, and F. Schauer (2004) Oxidation and ring cleavage of dibenzofuran by the filamentous fungus *Paecilomyces lilacinus. Arch. Microbiol.* **182**: 51–59.

Guha, S., and P.R. Jaffe (1996) Bioavailability of hydrophobic compounds partitioned into the micellar phase of nonionic surfactants. *Environ. Sci. Technol.* **30**: 1382–1391.

Guha, S., P.R. Jaffe, and C.A. Peters (1998) Bioavailability of mixtures of PAHs partitioned into the micellar phase of nonionic surfactant. *Environ. Sci. Technol.* **32**: 2317–2324.

Hamdy, M.K., and J.A. Gooch (1986) Uptake, retention, biodegradation, and depuration of PCBs by organisms. In: *PCBs and the Environment*, J.S. Weid, ed. CRC Press, Boca Raton, FL, Vol. II, pp. 63–88.

Hammel, K.E. (1992) Oxidation of aromatic pollutants by lignin-degrading fungi and their extracellular peroxidases. In: *Metal Ions in Biological Systems*, Vol. 28, *Degradation of Environmental Pollutants by Microorganisms and Their Metalloenzymes*, H. Sigel and A. Sigel, eds. Marcel Dekker, New York, pp. 41–60.

Hammer, E., and F. Schauer (1997) Fungal hydroxylation of dibenzofuran. *Mycol. Res.* **101**: 433–436.

Hammer, E., D. Krowas, A. Schafer, M. Specht, W. Francke, and F. Schauer (1998) Isolation and characterization of a dibenzofuran-degrading yeast: identification of oxidation and ring cleavage products. *Appl. Environ. Microbiol.* **64**: 2215–2219.

Hansen, J. (1998) GeoMelt In Situ Vitrification. Seminar on In Situ Thermal Treatment, Dec. 16, 1998, Atlanta, GA. EPA Technology Innovation Office, OSWER, Washington, DC.

Harms, H., M. Bokern, M. Kolb, and C. Bock (2003) Transformation of organic contaminants by different plant systems. In: *Phytoremediation: Transformation and Control of Contaminants*, S.C. McCutcheon and J.L. Schnoor, eds. Wiley-Interscience, Hoboken, NJ, pp. 285–216.

Hickey, W.J. (1996) Bioremediation of polychlorinated biphenyls (PCBs) in soil with microbial inoculants. *Int. Biodeterior. Biodegrad.* **37**: 245–246.

Hiratsuka, N., M. Oyadomari, H. Shinohara, H. Tanaka, and H. Wariishi (2005) Metabolic mechanisms involved in hydroxylation reactions of diphenyl compounds by the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Biochem. Eng. J.* **23**: 241–246.

Huang, S., and G.G. Gibson (1992) Species and congener specific induction of hepatic cytochrome P450 4A by PCBs. *Biochem. Pharmacol.* **43**: 637–639.

Hundt, K., U. Jonas, E. Hammer, and F. Schauer (1999) Transformation of diphenyl ethers by *Trametes versicolor* and characterization of ring cleavage products. *Biodegradation* **10**: 279–286.

Jackman, S.A., C.J. Knowles, and G.K. Robinson (1999) SACRED: a novel catalytic process for the environmental remediation of polychlorinated biphenyls (PCBs). *Chemosphere* **38**: 1889–1900.

Jensen, S. (1966) Report of a new chemical hazard. *New Sci.* **32**: 612.

Jonas, U., E. Hammer, E.T.K. Haupt, and F. Schauer (2000) Characterization of coupling products formed by biotransformation of biphenyl and diphenyl ether by the white rot fungus *Pycnosporus cinnabarinus. Arch. Microbiol.* **174**: 393–398.

Kamei, I., and R. Kondo (2005) Biotransformation of dichloro-, trichloro-, and tetrachlorodibenzo-*p*-dioxin by the white-rot fungus *Phlebia lindtneri. Appl. Microbiol. Biotechnol.* **68**: 560–566.

Keil, J.E., L.E. Priester, and S.H. Sandifer (1971) Polychlorinated biphenyl (aroclor 1242 ®): effects of uptake on growth, nucleic acids, and chlorophyll of a marine diatom. *Bull. Environ. Contam. Toxicol.* **6**: 156–159.

Kern, H.W. (1989) Improvement in the production of extracellular lignin peroxidase by *Phanerochaete chrysosporium*: effect of solid manganese(IV) oxide. *Appl. Microbiol. Biotechnol.* **32**: 223–234.

Kimbrough, R.D. (1995) Polychlorinated-biphenyls (PCBs) and human health: an update. *Crit. Rev. Toxicol.* **25**: 133–163.

Koller, G., M. Moder, and K. Czihal (2000) Peroxidation degradation of selected PCB: a mechanistic study. *Chemosphere* **41**: 1827–1834.

Krcmar, P., and R. Ulrich (1998) Degradation of polychlorinated biphenyl mixtures by the lignin-degrading fungus *Phanerochaete chrysosporium. Folia Microbiol.* **43**: 79–84.

Kubatova, A., M. Matucha, P. Erbanova, C. Novotny, V. Vlasakova, and V. Sasek (1998) Investigation into PCB degradation using uniformly ^{14}C-labeled dichlorobiphenyl. *Isotop. Environ. Health Stud.* **34**: 325–334.

Kubatova, A., P. Erbanova, I. Eichlerova, L. Homolka, F. Nerud, and V. Sasek (2001) PCB congener selective biodegradation by the white rot fungus *Pleurotus ostreatus* in contaminated soil. *Chemosphere* **43**: 207–215.

Kurek, B., and E. Odier (1990) Influence of lignin peroxidase concentration and localization in lignin biodegradation by *Phanerochaete chrysosporium. Appl. Microbiol. Biotechnol.* **34**: 264–270.

Lange, J., E. Hammer, M. Specht, W. Francke, and F. Schauer (1998) Biodegradation of biphenyl by the ascomycetous yeast *Debaryomyces vanrijiae. Appl. Microbiol. Biotechnol.* **50**: 364–368.

Layton, A.C., C.A. Lajoie, J.P. Easter, M. Muccini, and G.S. Sayler (1998) An integrated surfactant solubilization and PCB bioremediation process for soils. *Bioremed. J.* **2**: 43–56.

Legrini, O., E. Oliveros, and A.M. Braun (1993) Photochemical processes for water treatment. *Chem. Rev.* **93**: 671–698.

Madsen, E.L. (2003) *Report on Bioavailability of Chemical Wastes with Respect to the Potential for Soil Bioremediation*. EPA/600/R-03/076. Office of Research and Development, U.S. Environmental Protection Agency, Washington, DC.

Martinez, A.T. (2002) Molecular biology and structure–function of lignin-degrading heme peroxidases. *Enzyme Microb. Technol.* **30**: 425–444.

Mobley, D.P., H.L. Finkbeiner, S.H. Lockwood, and J. Spivack (1993) Synthesis of 3-arylmuconolactones using biphenyl metabolism in *Aspergillus. Tetrahedron* **49**: 3273–3280.

Mori, T., and R. Kondo (2002) Oxidation of chlorinated dibenzo-*p*-dioxin and diben-zofuran by white-rot fungus, *Phlebia lindtneri. FEMS Microbiol. Lett.* **216**: 223–227.

Murado, M.A., M.C. Tejedor, and G. Baluja (1976) Interactions between polychlori-nated biphenyls (PCBs) and soil microfungi: effects of Aroclor-1254 and other PCBs on *Aspergillus flavus* cultures. *Bull. Environ. Contam. Toxicol.* **25**: 768–774.

Nakamiya, K., S. Hashimoto, H. Ito, J.S. Edmonds, A. Yasuhara, and M. Morita (2005) Degradation of dioxins by cyclic ether degrading fungus, *Cordyceps sinen-sis. FEMS Microbiol. Lett.* **248**: 17–22.

Novotny, C., B.R.M. Vyas, P. Erbanova, A. Kubatova, and V. Sasek (1997) Removal of PCBs by various white rot fungi in liquid cultures. *Folia Microbiol.* **42**: 136–140.

Novotny, C., P. Erbanova, V. Sasek, A. Kubatova, et al. (1999) Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi. *Biodegradation* **10**: 159–168.

OHM Remediation Service (1995) *Trial Burn Report for the Baird & McGuire Super-fund Site.* Contract DACW45-92-C-0047. OHM, Holbrook, MA.

Orihara, K., T. Yamazaki., T. Shinkyo, T. Sakaki, K. Inouye, A. Tsukamoto, J. Sugiura, and K. Shishido (2005) Rat cytochrome P450-mediated transformation of dichlo-rodibenzo-*p*-dioxins by recombinant white-rot basidiomycete *Coriolus hirsutus. Appl. Microbiol. Biotechnol.* **69**: 22–28.

Pieper, D.H. (2005) Aerobic degradation of polychlorinated biphenyls. *Appl. Micro-biol. Biotechnol.* **67**: 170–191.

Pignatello, J., and C. Guadalupe (1994) Degradation of PCBs by ferric ion, hydrogen peroxide and UV light. *Environ. Toxicol. Chem.* **13**: 423–427.

Quensen, J.F., III, S.A. Boyd, and J.M. Tiedje (1990) Dechlorination of four commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic micro-organisms from sediments. *Appl. Environ. Microbiol.* **56**: 2360–2369.

Rahuman, M.S.M.M., L. Pistone, F. Trifiro, and S. Miertus (2000) *Destruction Tech-nologies for Polychlorinated Biphenyls (PCBs)*. International Centre for Science and High Technology, United Nations Industrial Development Organization (ICS-UNIDO), Trieste, Italy.

Robinson, G.K., and M.J. Lenn (1994) The bioremediation of polychlorinated biphe-nyls (PCBs): problems and perspectives. *Biotechnol. Genet. Eng. Rev.* **12**: 139–188.

Rojas-Avelizapa, N.G., R. Rodriguez-Vazquez, F. Enriquez-Villanueva, J. Martinez-Cruz, and H.M. Poggi-Veraldo (1999) Transformer oil degradation by an indige-

nous microflora isolated from a contaminated soil. *Resour. Conserv. Recycl.* **27**: 15–26.

Ruiz-Aguilar, G.M.L., J.M. Fernandez-Sanchez, R. Rodriguez-Vazquez, and H. Poggi-Veraldo (2002) Degradation by white rot fungi of high concentrations of PCB extracted from a contaminated soil. *Adv. Environ. Res.* **6**: 559–568.

Sakaki, T., R. Shinkyo, T. Takita, M. Ohta, and K. Inouye (2002) Biodegradation of polychlorinated dibenzo-*p*-dioxins by recombinant yeast expressing rat CYP1A subfamily. *Arch. Biochem. Biophys.* **401**: 91–98.

Sasek, V., O. Volfova, P. Erbanova, B.R.M. Vyas, and M. Matucha (1993) Degradation of PCBs by white rot fungi, methylotrophic and hydrocarbon utilizing yeasts and bacteria. *Biotechnol. Lett.* **15**: 521–526.

Schauer, F., K. Henning, H. Pscheidl, R.M. Wittich, et al. (1995) Biotransformation of diphenyl ether by the yeast *Trichosporon beigelii* SBUG 752. *Biodegradation* **6**: 173–180.

Schultz, A., U. Jonas, E. Hammer, and F. Schauer (2001) Dehalogenation of chlorinated hydroxybiphenyls by fungal laccase. *Appl. Environ. Microbiol.* **67**: 4377–4381.

Schwartz, R.D., A.L. Williams, and D.B. Hutchinson (1980) Microbial production of 4,4′-dihydroxybiphenyl: biphenyl hydroxylation by fungi. *Appl. Environ. Microbiol.* **39**: 702–708.

Sedlak, D.L., and A.W. Andren (1991) Aqueous-phase oxidation of polychlorinated biphenyls by hydroxyl radicals. *Environ. Sci. Technol.* **5**: 1419–1427.

Seto, M., K. Nishibori, E. Masai, M. Fukuda, and Y. Ohdaira (1999) Degradation of polychlorinated biphenyls by a 'Maitake' mushroom, *Grifola frondosa. Biotechnol. Lett.* **21**: 27–31.

Sietmann, R., E. Hammer, M. Specht, C.E. Cerniglia, and F. Schauer (2001) Novel ring cleavage products in the biotransformation of biphenyl by the yeast *Trichosporon mucoides. Appl. Environ. Microbiol.* **67**: 4158–4165.

Smith, R.V., and J.P. Rosazza (1974) Microbial models of mammalian metabolism: aromatic hydroxylation. *Arch. Biochem. Biophys.* **161**: 551–558.

Smith, R.V., P.J. Davis, A.M. Clark, and S. Glover-Milton (1980) Hydroxylations of biphenyl by fungi. *J. Appl. Bacteriol.* **49**: 65–73.

Smith, R.V., P.J. Davis, A.M. Clark, and S.K. Prasatik (1981) Mechanism of hydroxylation of biphenyl by *Cunninghamella echinulata. Biochem. J.* **196**: 369–371.

Tabak, H.H., and R. Govind (1997) Protocol for determining the bioavailability and biokinetics of organic pollutants in dispersed, compacted and intact soil systems to enhance in situ bioremediation. *J. Ind. Microbiol. Biotechnol.* **18**: 330–339.

Takada, S., M. Nakamura, T. Matsueda, R. Kondo, and K. Sakai (1996) Degradation of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans by the white rot fungus *Phanerochaete sordida* YK-624. *Appl. Environ. Microbiol.* **62**: 4323–4328.

Tejedor, M.C., M.A. Murado, and G. Baluja (1979) Oxidative metabolism in *Saccharomyces cerevisiae* as affected by polychlorinated biphenyls. *Bull. Environ. Contam. Toxicol.* **22**: 439–448.

Thomas, D.R., K.S. Carswell, and G. Georgiou (1992) Mineralization of biphenyl and PCB by the white rot fungus *Phanerochaete chrysosporium. Biotechnol. Bioeng.* **40**: 1395–1402.

USEPA (1998) *New Protocol on Persistent Organic Pollutants.* Negotiated under the UN Economic Commission for Europe. Convention on long-range transboundary air pollution. U.S. Environmental Protection Agency, Washington, DC.

USEPA (1999) *Pesticide Use and Disposal.* Technical information packages. U.S. Environmental Protection Agency, Washington, DC.

Valli, K., H. Wariishi, and M.H. Gold (1992) Degradation of 2,7-dichlorodibenzo-*p*-dioxin by the lignin degrading basidiomycete *Phanerochaete chrysosporium. J. Bacteriol.* **174**: 2131–2137.

Varela, E., A.T. Martinez, and M.J. Martinez (2000) Southern blot screening for lignin peroxidase and aryl-alcohol oxidase genes in 30 fungal species. *J. Biotechnol.* **83**: 245–251.

Viney, I., and R.J.F. Bewley (1990) Preliminary studies on the development of a microbiological treatment for polychlorinated biphenyls. *Arch. Environ. Contam. Toxicol.* **19**: 789–796.

Voice, T.C., M.J. Szafranski, M.A. Maraqa, and X. Zhao (1997) Elucidation of fundamental mechanisms controlling bioavailability. In: *Proceedings of the Fourth International In Situ and On-Site Bioremediation Symposium*, Apr. 28–May 1, B.C. Alleman and A. Leeson, eds. Battelle Press, Columbus, OH, Vol. 5, pp. 657–662.

Vyas, B.R.M., V. Sasek, M. Matucha, and M. Bubner (1994) Degradation of 3,3′,4,4′-tetrachlorobiphenyl by selected white rot fungi. *Chemosphere* **28**: 1127–1134.

Wallnofer, P.R., G. Engelhardt, S. Safe, and O. Hutzinger (1973) Microbial hydroxylation of 4-chlorobiphenyl and 4,4′-dichlorobiphenyl. *Chemosphere* **2**: 69–72.

Wiseman, A., J.A. Gondal, and P. Sims (1975) 4′-Hydroxylation of biphenyl by yeast containing cytochrome P-450: radiation and thermal stability, comparisons with liver enzyme (oxidized and reduced forms). *Biochem. Soc. Trans.* **3**: 278–281.

Wright, M.A., C.J. Knowles, J. Stratford, S.A. Jackman, and G.K. Robinson (1996) The dechlorination and degradation of Aroclor 1242. *Int. Biodeterior. Biodegrad.* **38**: 61–67.

Yadav, J.S., J.F. Quensen III, J.M. Tiedje, and C.A. Reddy (1995) Degradation of polychlorinated biphenyl mixtures (Aroclors 1242, 1254, and 1260) by the white rot fungus *Phanerochaete chrysosporium* as evidenced by congener-specific analysis. *Appl. Environ. Microbiol.* **61**: 2560–2565.

Zeddel, A., A. Majcherczyk, and A. Huttermann (1993) Degradation of polychlorinated biphenyls by white-rot fungi *Pleurotus ostreatus* and *Trametes versicolor* in a solid state system. *Toxicol. Environ. Chem.* **40**: 255–266.

6

FUNGAL DEGRADATION OF PESTICIDES

6.1   INTRODUCTION

Synthetic pesticides have been known since 1939, when the insecticidal properties of DDT were discovered (Tessier, 1982). Pesticides are used extensively related to agriculture, animals, and humans to protect the public health. Soon after the discovery of various severe deleterious effects on animals and humans, several pesticides were banned for manufacture and use. The extensive use of pesticides has contributed to the contamination of many terrestrial and aquatic global ecosystems. Due to the extreme toxicity and persistence in the environment, they have been the target of research throughout the world for the past four decades.

Groundwater pollution is common in regions of the United States due to the impact of agricultural practices. As a result of such practices, considerable quantities of pesticide residues can be found in the soil and have the potential of leaching to the underlying groundwater. Leaching depends on the sorption and microbial degradation of pesticides in the soil. The chemical characteristics of the pesticides also influence the sorption. In brief, soil properties, especially the organic fraction, affects the sorption and eventually, the potential for leaching. Current knowledge of the microbial metabolism of pesticides in the soil is limited. In addition, degradation appears to be the only detoxification process of pesticides in the environment. Several review articles on the fate of microbial degradation of pesticides in the environment were

published between 1970s and 1990s. The degradation of pesticides by fungi has also been discussed (Singh et al., 1991; Maloney, 2001).

Little is understood on the role of yeasts and fungi in the detoxification of pesticides as compared to bacteria. Soil type, pH, organic matter, fungal biomass, moisture, aeration, and so on, are important factors affecting biodegradation. Complete degradation of pesticides may occur through the Krebs cycle. Four major possibilities for microbial transformation of pesticides have been postulated (Bollag, 1974). In fungi, the best known transformations take place through cometabolism. The important biochemical reactions in the fungal degradation of pesticides are alkylation, dealkylation, amide or ester hydrolysis, dehalogenation, dehydrogenation, hydroxylation, ether cleavage, ring cleavage, oxidation, reduction, condensation, and conjugate formation.

6.2   CLASSIFICATION

It is difficult to classify pesticides into different categories. However, based on their applications in agriculture, animals, and humans, pesticides are divided into three categories: insecticides, herbicides, and fungicides. Insecticides, herbicides, and fungicides are further classified into different categories based on chemical composition and organic grouping. A global compendium of pesticides has been edited by Tomlin (2000). This includes the chemical structure, discipline and class, nomenclature, general and trade names, classes, physical chemistry details, patents, modes of action, toxicology profiles, ecotoxicity data, and environmental fate information.

6.3   BIOSENSORS FOR DETECTION OF PESTICIDES

Classical analytical methods for pesticide analysis include high-performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC), enzyme-linked immunosorbant assay (ELISA), and spectrophotometric methods. Of these, the most widely used method is high-resolution gas chromatography with an element-specific detector. Commonly used GC detectors include the electron capture detector (ECD), nitrogen phosphorus detector (NPS), and flame photometric detector (FPD) for the detection of chlorinated, nitrogen-containing, and organophosphorus pesticides, respectively. Electrolytic conductivity detectors (ELCDs) and atomic emission detectors (AEDs) are also used. Quantification of pesticides is measured by GC-MS, and this has become a common method of identification and confirmation. However, these analytical methods are time consuming and expensive, as they require sample preconcentration and sometimes derivation.

Biosensors are analytical devices responsible for converting biochemical reactions into electronic signals that can be processed, transmitted, and measured. Biosensors usually consist of a bioactive material such as enzyme, antibody, or binding protein, whole cell, or organelle in intimate contact with a transducer. These are analyte-specific, rapid, reliable, portable, inexpensive, sensitive, and automated devices. The transducers may be amperometric, potentiometric, fluorimetric, piezoelectric, fiber optic, chemiluminescence, or based on semiconductor or ion-sensitive field-effect transistor techniques. Biosensors can provide faster on-site results for the monitoring of sources of pesticides in the environment. Based on the type of bioactive material used, biosensors can be classified into four categories: (1) enzyme-based biosensors, (2) immunosensors, (3) receptor-based biosensors, and (4) cell/tissue-based biosensors. Various types of biosensors for the detection of pesticides have been described by Rekha et al. (2000).

In biosensors, flow injection analysis (FIA) systems are generally employed for continuous analysis. The quality of a biosensor depends on certain technical and functional features, such as selectivity, sensitivity, and detection limit, linearity of signal response, stability and operating life, response time, and recovery time (Alexander, 1990). Several factors are known to influence the performance of biosensors. These factors are enzyme concentration, immobilization of enzymes, influence of immobilization on enzyme kinetics, influence of substrate concentration, and the influence of pH, temperature, and solvents. Several biosensors have been developed in Germany for the detection of pesticides in soil and water samples (Anon., 1996).

Enzyme-based biosensors involve enzyme-catalyzed redox reactions, forming a product or the disappearance of electroactive substrate. Cholinesterase and tyrosinase are the principal enzymes used for the detection of pesticides. A ferromagnetic biosensor has also been used for the assay of organophosphorus pesticides (Khavkin and Khavkin, 1996). A photothermal biosensor containing acetyl cholinesterase has been employed for the detection of paraoxon, chlorpyrifos, and diazinon in tap water at a low concentration of 0.2 ng (Pogacnik and Franko, 1999). Atrazine and carbamate pesticides can be determined by tyrosinase inhibition using an amperometric biosensor (Besombes et al., 1995).

Immunosensors are devices that detect antigen binding to a specific antibody by coupling the immunochemical reaction to the surface of a transducer. Developments in immunosensors have focused interest on commercializing immunosensors in the clinical, food, and environmental areas (Gizeli and Lowe, 1996). These biosensors offer both the high specificity of immunological methods such as radioimmunoassay (RIA) and ELISA and low detection limits of electrochemical systems. Antibodies with a piezoelectric-based transducer are employed as a biological component in immunosensors. These methods can attain good sensitivity for the determination of pesticides in drinking water. Highly sensitive quartz crystal immunosensors are employed for the multisample detection of herbicides in water.

Atrazine has been detected in the concentration range 0.001 to 1 ng/ml and the detection limit 0.001 ng/ml (Yokoyama et al., 1995). A rapid assay based on an immunoenzyme electrode and peroxidase conjugates have been developed for the determination of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Dzantiev et al., 1996). Triazine herbicides can be determined by an atrazine–BSA–Eu(III)–chelate conjugate as a fluorescence marker to detect analyte–antibody reaction (Wortberg et al., 1995). A range of commercially available immunosensors are available for the detection of pesticides in soil and water (Kramer, 1996).

A new label, laccase from *Coriolus hirsutus*, has been used for solid-phase enzyme-linked immunosorbent assays of 2,4-D (Zherdev et al., 1999). This system allowed detection of 2,4-D in concentrations to 10 to 20 ng/ml within 1.5 to 2 hours. Isolated plant or animal receptors can be employed to devise receptor-based biosensors. Cell- and tissue-based biosensors are more promising for the monitoring of pollutants. A mushroom-based biosensor has been developed for the monitoring of diethyldithiocarbamates (Wang et al., 1996). Different types of amperometric biosensors based on *Synechococcus* cyanobacteria have been discussed for use in determining the presence pesticides, phenolics, and heavy metals at low concentration of 20 ppm in surface water and groundwater (Rawson and Gaisford, 1990). A lipid membrane-based biosensor is a rapid technique for the analysis of mixtures for triazine herbicides (Nikolelis and Siontorou, 1996). Multianalyte measurement (measurement of a number of pesticides of the same class and different classes) is a big problem in the commercialization of pesticide biosensors. It is important to develop and integrate software into the biosensor-based analytical systems.

6.4   FUNGAL DEGRADATION OF INSECTICIDES

The application of insecticides enjoyed popularity in tropical countries during the middle of the twentieth century. In the past decades their use has declined, due to various known levels of toxicity to human health and the environment. Table 6.1 notes the rates of degradation and mineralization and metabolic products of various insecticides by fungi.

6.4.1   Chlorinated Compounds

The chlorinated insecticides, including DDT, aldrin, dieldrin, heptachlor, endrin, chlordane, and endosulfan, are of major environmental concern. DDT [1,1,1-trichloro-2,2-bis-(4-chlorophenyl)ethane] is an insecticide used extensively since the 1940s and banned in most industrialized nations in 1972. The bacterial degradation of DDT involves reductive dechlorination, oxidation, decarboxylation, and subsequently, ring cleavage. The bioremediation of DDT-contaminated soils has been described by Foght et al. (2001).

TABLE 6.1 Fungal Degradation of Insecticides

Fungus	Insecticide Conc.	Medium	Degradation/ Mineralization Rate (%)	Metabolic Products	Duration (days)	Reference
Phanerochaete chrysosporium Pleurotus ostreatus Phellinus weirii Polyporus versicolor	[^{14}C]DDT, 1.25 nM	N-deficient cultures	10 as $^{14}CO_2$ 5.9 as $^{14}CO_2$ 5.7 as $^{14}CO_2$ 5.3 as $^{14}CO_2$	Dicofol, FW-152, DBP	30	Bumpus and Aust, 1987
P. chrysosporium	[^{14}C]DDE, 10 nM	Nutrient N-limited cultures	6 as $^{14}CO_2$	DBP and unidentified compounds	60	Bumpus et al., 1993a
P. chrysosporium	[ring-^{14}C] Methoxychlor, 10 μM	N-limited medium		1,1-Dichloro-2,2-bis (4-methoxyphenyl)ethane, 2,2,2-trichloro-1,1-bis (4-methoxyphenyl)ethanol, 2,2-dichloro-1,1-bis (4-methoxyphenyl)ethanol	7	Grifoll and Hammel, 1997
P. chrysosporium	[^{14}C]Lindane, 54 mCi/mM [^{14}C]Chlordane, 5.91 mCi/mM	Liquid culture and soil–corncob matrix	23.4 as $^{14}CO_2$ 22.8 as $^{14}CO_2$ 9.4 as $^{14}CO_2$ 15 as $^{14}CO_2$		30 60 30 60	Kennedy et al., 1990
Trichoderma harzianum	Endosulfan, 1 mg/l Dieldrin, 1 mg/l DDT, 1 mg/l	Sucrose-mineral medium	39 26 17	Endosulfan sulfate, endosulfan diol	13	Katayama and Matsumura, 1993
P. chrysosporium	Endosulfan, 1 μg/ml	C-deficient N-deficient N-rich	>95 >90 61	Endosulfan sulfate, endosulfan diol, endosulfan hydroxyether, endosulfan dialdehyde	50	Kullman and Matsumura, 1996

TABLE 6.1 Continued

Fungus	Insecticide Conc.	Medium	Degradation/ Mineralization Rate (%)	Metabolic Products	Duration (days)	Reference
P. chrysosporium	Lindane, 0.27 μM	Liquid medium containing VA	3.9 as $^{14}CO_2$	Tetrachlorocyclohexene, tetrachlorocyclohexene epoxide, tetrachlorohexenol	14	Mougin et al., 1996
White-rot fungus DSPM95	Lindane, 5 and 10ppm	Synthetic mineral medium containing Tween and VA	82		30	Tekere et al., 2002
P. chrysosporium	1 and 2 ppm [^{14}C]Chlorpyrifos, 3.5 μM [^{14}C]Fonofos, 8.5 μM [^{14}C]Terbufos, 1.4 μM	Packed-bed reactors Nutrient N-limited cultures	81 27.5 12.2 26.6 as $^{14}CO_2$		5 18	Bumpus et al., 1993b
Coriolus versicolor *Hypholoma fasciculare* *Stereum hirsutum*	Chlorpyrifos, 20μg/g	Biobed matrix	36 29 6		42	Bending et al., 2002
P. chrysosporium	Hydramethylnon, 70μM	N-deficient conditions		p-(Trifluoromethyl)cinnamic acid, p-(trifluoromethyl)benzoic acid	10	Abernathy and Walker, 1993

Certain fungi, such as *Mucor alternans* (Anderson and Lichtenstein, 1971), *Fusarium oxysporum* (Engst and Kujawa, 1968), and *Trichoderma viride* (Matsumura and Bousch, 1968), are known to degrade DDT for a long time. The pathways of degradation of DDT by bacteria and fungi appear to be similar. Ligninolytic fungi also have the ability to degrade DDT, and the major work has focused on *Phanerochaete chrysosporium*. A pathway for the degradation of DDT by the white-rot fungus *P. chrysosporium* has been postulated by Bumpus and Aust (1987) and Bumpus et al. (1993a) and is depicted by Foght et al. (2001). The rate and extent of mineralization of [^{14}C]dicofol and [^{14}C]methoxychlor, a [^{14}C]DDT analog, are substantially higher than those of [^{14}C]DDT. The fungus is known to produce lignin per-oxidase (LiP), which mineralizes DDT. Formation of DDD [1,1-dichloro-2,2-bis-(4-chlorophenyl)ethane] occurs during initial nonligninolytic phase and is subsequently degraded. This suggests the formation of DDD by a mechanism different from a lignin-degrading system. The mineralization of [^{14}C]DDE is slower than that of DDT (Bumpus et al., 1993a). The extent of mineralization of DDT by *P. chrysosporium* depends on the source of carbon (Fernando et al., 1989). Cellulose and starch prove better carbon sources in supporting [^{14}C]DDT mineralization, and $^{14}CO_2$ evolution stops on exhaus-tion of the carbon supply. The role of LiP is still not fully understood in the mineralization of DDT. However, the mineralization of DDT coincides with the production of LiP. LiP inhibitors such as ethylenediaminetetraacetic acid (EDTA) and tetramethylethylenediamine (TEMED) also inhibit DDT min-eralization. It appears that DDT is degraded through cooxidation by a media-tor such as veratryl alcohol (Khindaria et al., 1995). It is also possible that LiPs may oxidize DDT metabolites other than DDT.

P. chrysosporium dechlorinates [ring-^{14}C]methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] to three metabolites (Grifoll and Hammel, 1997). All three metabolites were mineralized. The fungus converted both 1-dechloro and 2-hydroxy derivatives to a 1-dechloro-2-hydroxy derivative, suggesting that dechlorination does not precede hydroxylation, or vice versa. *P. chrysosporium* caused significant bioconversion of [^{14}C]aldrin, [^{14}C]dieldrin, [^{14}C]heptachlor, and [^{14}C]mirex, but these compounds were poorly mineral-ized (Kennedy et al., 1990). Species of soil fungi (e.g., *Fusarium*, *Penicillium*, *Rhizopus*, and *Trichoderma*) have the ability to convert heptachlor to hepta-chlor epoxide. Methods have been developed for *P. chrysosporium* to inocu-late soils contaminated with organochlorines (Lestan et al., 1996).

Trichoderma harzianum degrades the cyclodiene insecticide endosulfan (1,4,5,6,7,7-hexachloro-5-norbornene-2,3-dimethanol cyclic sulfite) under various nutrient media throughout its growth stages (Katayama and Mat-sumura, 1993). The degradation of endosulfan is catalyzed by the oxidation of endosulfan to endosulfan sulfate followed by hydrolysis to endosulfan diol. Exogenous addition of nicotinamide adenine dinucleotide phosphate (NADPH) enhances endosulfan metabolism. Formation of the initial meta-bolic product of endosulfan sulfate is catalyzed by the major oxidative enzyme

system (oxidase) in *T. harzianum*. Endosulfan sulfate is produced as a major metabolite due to endosulfan degradation by many soil fungi (Martens, 1972). Other metabolites (e.g., endoether, endohydroxyether, chlorendic acid, and endolacetate) have also been identified. Soil fungi can be classified into two categories: one type that accumulates endosulfan sulfate and a second type that converts endosulfan sulfate metabolically to endosulfan diol (El-Zorgani and Omer, 1974; Martens, 1976). None of the species of *Trichoderma* is known as an endosulfan degrader other than *T. harzianum*, which belongs to the second type of fungi. Hydrolysis of endosulfan sulfate to endosulfan diol can be catalyzed by hydrolases (e.g., sulfatase). It appears that hydrolyzing enzymes are present in the second type of fungi, in addition to oxidative enzymes. The metabolic pathway for endosulfan metabolism by *P. chryso-sporium* BU-1 is shown in Figure 6.1 (Kullman and Matsumura, 1996). Initially, endosulfan is either oxidized to endosulfan sulfate, a terminal end product, or hydrolyzed to non-sulfur-containing endosulfan metabolites. Initial hydrolysis of endosulfan produces endosulfan diol, which is further oxidized to endosulfan hydroxyether followed by the formation of either endosulfan lactone or the putative endosulfan dialdehyde. Piperonyl butox-ide, a cytochrome P450 inhibitor, inhibits the oxidation of endosulfan to endosulfan sulfate and enhances the hydrolysis of endosulfan to endosulfan
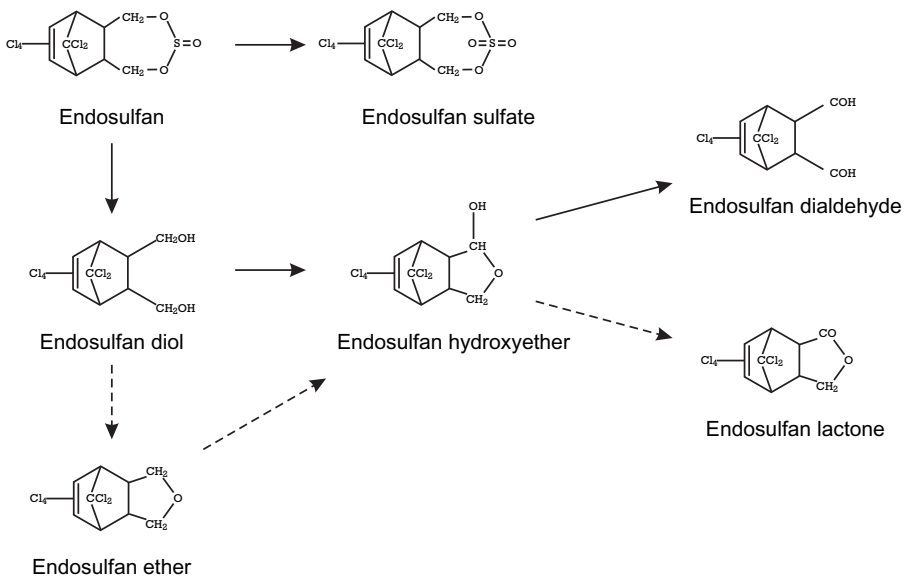
Figure 6.1 Pathways for the metabolism of endosulfan by *Phanerochaete chryso-sporium* BU-1. Solid arrows, major metabolic pathways; dashed arrows, minor metabolic pathways. [Reprinted from Kullman and Matsumura (1996), copyright © with permission from the American Society for Microbiology and the authors.]

diol. Thus, the metabolism of endosulfan involves two divergent pathways (e.g., hydrolytic and oxidative).

Lindane (1α,2α,3β,4α,5α,6β-hexachlorocyclohexane, γ-HCH) has been used extensively in the world despite its persistence in the environment. *P. chrysosporium* can biotransform [^{14}C]lindane in liquid culture at 25°C-air conditions, resulting in the formation of some polar metabolites and the release of [^{14}C]CO$_2$ (Mougin et al., 1996). Transformation of tetrachlorocyclohexene as a substrate in liquid culture also confirms the identity of three polar degradation products and carbon dioxide. Lindane appears to be completely mineralized or converted to water-soluble products in both batch and immobilized packed-bed bioreactor systems by the subtropical white-rot fungus DSPM95 (Tekere et al., 2002). MnP and laccase are produced in both systems, but high enzyme levels are not sustained for longer periods in the packed-bed system. Lindane was degraded in agitated liquid cultures by *Pleurotus ostreatus* using the central composite design as a second-order methodology (Rigas et al., 2005). The maximum biodegradation of lindane was 25.8 mg per gram of initial lindane and per gram of biomass at 1.89 g/l nitrogen content, 12.45 days, and 24°C.

6.4.2 Organophosphorus Compounds

The organophosphorus insecticides are used extensively in agriculture and are easily degraded by bacteria in the environment. A number of them have half-lives on the order of several months and appear to be moderately persistent. The organophosphorus insecticides include malathion, parathion, diazion, fenitrothion, fenitrooxon, disulfoton, fonofos, and bromophos. Malathion and parathion were used widely in the past, due to the broad spectrum and high insecticide activities and are of great environmental concern. Hydrolysis is the principal mechanism of degradation of organophosphorus insecticides, and it can be both chemical and biological in nature. Transformation of malathion to β-monoacid and dicarboxylic acid occurs due to carboxyesterase activity by *Aspergillus oryzae* (Lewis et al., 1975). These metabolites were subsequently converted to thio- and inorganic phosphates. *Penicillium waksmanii* isolated from flooded acid sulfate soil has the capacity to degrade parathion to aminoparathion (Rao and Sethunathan, 1974). The chlorinated pyridinyl ring of chlorpyrifos and the phenyl ring of fonofos undergo cleavage during biodegradation by *P. chrysosporium* (Bumpus et al., 1993b). Several species of a mixed population of fungi, such as *Alternaria alternata*, *Cephalosporium* sp., *Cladosporium cladosporioides*, *Cladorrhinum brunnescens*, *Fusarium* sp., *Rhizoctonia solani*, and *Trichoderma viride*, reveal the degradation of chlorpyrifos in liquid culture (Al-Mihanna et al., 1998). The degradation of chlorpyrifos proves more efficient by mixed populations than by pure cultures of fungi. Fenitrothion and fenitrooxon are degraded enzymatically by *T. viride* in the presence of alternative nutrients (Baarschers and Heitland, 1986). Of three white-rot fungi, *Coriolus versicolor* is the best

performer to degrade chlorpyrifos in a biobed matrix (Bending et al., 2002). The degradation by fungi of organophosphorus compounds of lesser environmental concern (e.g., diazion, disulfoton, fonofos, and bromophos) has also been described (Singh et al., 1991).

6.4.3   Miscellaneous Compounds

Hydramethylnon (HMN) is an amidinohydrazone-type insecticide used for the control of red introduced fire ants (RIFA). Certain soil fungi (Abernathy and Walker, 1993) can decompose HMN easily. HMN is degraded in soil with a half-life of 14 to 25 days. The disappearance of HMN is correlated with the LiP activity of *P. chrysosporium*. However, addition of nutrient-N suppresses both HMN disappearance and LiP activity. Two metabolites, *p*-(trifluoromethyl)cinnamic acid (TFCA) and *p*-(trifluoromethyl)benzoic acid (TFBA), are produced as a result of degradation of HMN by *P. chrysosporium*. Further metabolism by *P. chrysosporium* converts TFCA to TFBA, which is subsequently metabolized via the *meta*-fission pathway. Several keto-acids are produced in cultures of *P. chrysosporium* incubated with TFBA or TFCA. The liberation of fluoride from HMN has not been detected. A tentative pathway for the degradation of HMN by *P. chrysosporium* has been described. The degradation by fungi of carbamate compounds of lesser environmental concern (e.g., carbaryl, carbofuran, and aldicarb) has also been described (Singh et al., 1991). Salama (1998) showed the degradation of carbofuran by pure cultures of *Aspergillus niger* and *Fusarium graminearum*.

6.5   FUNGAL DEGRADATION OF HERBICIDES

More than 2500 pesticides are currently in use in the world (Anon., 1998). Over 150 chemicals are used as herbicides throughout the world (Singh et al., 1991). The herbicides constitute 45%, followed by insecticides (35%) and fungicides (15%), of all the pesticides used in the developed countries. Their count is different in tropic and subtropic regions, where the use of insecticides is highest. Most of the literature on the detoxification of herbicides by microorganisms (i.e., fungi, bacteria, and actinomycetes) is based on studies in developed countries. The metabolic pathways for the degradation of herbicides by these microorganisms are partially known. However, the role of fungi in the detoxification of herbicides has been known for several decades. Table 6.2 notes the rates of degradation and mineralization and metabolic products of various herbicides by fungi.

6.5.1   Phenoxyalkanoate Compounds

A plethora of literature is available on the microbial degradation of herbicides of phenoxyalkanoic acid derivatives. However, less attention is given to the

TABLE 6.2 Fungal Degradation of Herbicides

Fungus	Herbicide Conc.	Medium	Degradation/ Mineralization Rate (%)	Metabolic Products	Duration (days)	Reference
Phanerochaete chrysosporium	[^{14}C]2,4,5-T, 6.0nM	Nutrient N-limited culture 2,4,5-T-contaminated soil amended with ground corncobs	62 as $^{14}CO_2$ 32.5 as $^{14}CO_2$	Water-soluble compounds	30	Ryan and Bumpus, 1989
P. chrysosporium	[U-(ring)-^{14}C]2,4-D (10^5 cpm) along with 5 mg of unlabeled 2,4-D per liter	Malt extract medium	12.5		20	Yadav and Reddy, 1993
	[^{14}C]2,4-D, 5 mg/l and/or 2,4,5-T, 10 mg/l	Malt extract medium	46.5 as $^{14}CO_2$		36	
Cunninghamella elegans	2,4-D, 20 mg/l	Galzy and Slonimski (GS) medium	33		5	Vroumsia et al., 1999
C. echinulata			47			
Rhizoctonia solani			60			
Verticillium lecanii			56			
Aspergillus penicilloides	2,4-D, 100 mg/l	GS synthetic liquid medium	52	2,4-DCP	7	Vroumsia et al., 2005
Mortierella isabellina	2,4-D, 100 mg/l		46	2,4-DCP		
Ceriporiopsis subvermispora	[U-^{14}C-(ring)-labeled] Alachlor, 18 ppm	Malt extract medium supplemented with wood chips	14 as $^{14}CO_2$	Polar and nonpolar metabolites	122	Ferrey et al., 1994

TABLE 6.2 Continued

Fungus	Herbicide Conc.	Medium	Degradation/ Mineralization Rate (%)	Metabolic Products	Duration (days)	Reference
Phlebia tremellosa						
C. elegans	[¹⁴C]Alachlor, 20 mg	Sabouraud dextrose broth	12 as ¹⁴CO2 / 98.6	Four metabolites	4	Pothuluri et al., 1993
Fusarium oxysporum	Butachlor, 50 ppm	Potato dextrose broth	70	Three metabolites	12	Chakraborty and Bhattacharyya, 1991
F. solani			85	30–32 Metabolites		
R. solani	Chlortoluron, 100 mg/l	GS medium	100	Unidentified products	15	Vroumsia et al., 1996
	Diuron, 20 mg/l		100		10	
	Isoproturon, 100 mg/l		100		10	
Bjerkandera adusta	Chlortoluron, 100 mg/l	GS medium	98		15	Khadrani et al., 1999
	Diuron, 20 mg/l		92			
	Isoproturon, 100 mg/l		88			
Sordaria superba	Linuron, 100 mg/l	Liquid culture medium	25		5	Bordjiba et al., 2001
Botrytis cinerea	Linuron, 100 mg/l		91			
	Metobromuron, 100 mg/l		92			
Rhizopus oryzae	Metobromuron, 100 mg/l		40			
Absidia fusca			47			
Alternaria solani	Metamitron, 100 mg/l		11			
Drechslera australiensis			10			
A. fusca			16			
B. cinerea	Metribuzin, 100 mg/l		61			
S. superba			64			
A. fusca			95			

Organism	Substrate, concentration	Medium	%		Products	Reference
Oxysporus sp.	Chlortoluron, 100 mg/l Diuron, 20 mg/l Isoproturon, 100 mg/l	GS medium	76 64 56	5		Bending et al., 2002
Coriolus versicolor *Hypholoma fasciculare* *Stereum hirsutum*	Diuron, 10 mg/l	Liquid culture	100 71	42		
C. versicolor *Hypholoma fasciculare* *S. hirsutum*	Diuron, 20 μg/g	Biobed matrix	80 52 16	42		
H. fasciculare *S. hirsutum* *C. versicolor* *S. hirsutum* *H. fasciculare*	Terbuthylazine, 10 mg/l Atrazine, 10 mg/l	Liquid culture	74 97 88 86 58 58	42		
H. fasciculare *C. versicolor* *C. versicolor* *S. hirsutum* *H. fasciculare*	Terbuthylazine, 20 μg/g Atrazine, 20 μg/g	Biobed matrix	37 40 51 57 61	42		
P. chrysosporium	Atrazine, 2 μM	Growth medium	48	4	Deethylatrazine, deisopropylatrazine, hydroxyatrazine, deethylhydroxyatrazine	Mougin et al., 1994
Pleurotus pulmonarius	Atrazine, 149 μg/g	Solid-state fermentation	74	42	CAIT, CEAT, CEIOT, CAAT, OEIT, OAIT, OEAT, OAAT,	Masaphy et al., 1996a
P. pulmonarius	Atrazine, 5 mg/l	Liquid culture and Mn(II)		1	CAIT, CEAT, CEIOT, deethyldeisopropylatrazine	Masaphy et al., 1996b

fungal degradation of these compounds. *Penicillium megasporum* and *Penicillium* sp. utilize 2,4-D as a carbon source (Torstensson et al., 1975). A small amount (2 to 3%) of $^{14}CO_2$ evolves from [^{14}C]-2,4-D by *Hendersonula toruloidea* (Wolf and Martin, 1976). A strain of *Aspergillus niger* dechlorinates 2,4-D prior to ring cleavage (Sahasrabudhe and Modi, 1987).

Extensive degradation of [^{14}C]-2,4,5-T by *P. chrysosporium* can occur in nitrogen-limited aqueous cultures and in [^{14}C]-2,4,5-T-contaminated soil amended with ground corncobs (Ryan and Bumpus, 1989). The mass balance analysis shows the formation of water-soluble metabolites. However, HPLC and TLC of methylene chloride–extractable material reveal the presence of polar and nonpolar metabolites. Only about 5% of the [^{14}C]-2,4,5-T in the cultures remains undegraded. The fungus mineralizes [^{14}C]-2,4,5-T–contaminated nonsterile soil amended with ground corncobs, which suggests the possibility of development of a practical treatment system to detoxify 2,4,5-T-contaminated soil and water.

Wild type and a peroxidase-negative mutant of *P. chrysosporium* can mineralize 2,4-D and mixtures of 2,4-D and 2,4,5-T (Yadav and Reddy, 1993). LiP and MnP are not required for 2,4-D mineralization. Higher rates of mineralization occur when these compounds are tested in combination than when tested alone. A mixture of 2,4-D and 2,4,5-T leads to simultaneous mineralization of 46.5%, and there is no mutual inhibition of degradation. The efficacy of degradation of 2,4-D by four fungi is regulated by culture parameters (Vroumsia et al., 1999). An initial concentration of 20 mg/l of 2,4-D, low concentrations of glucose (5 g/l), and 2.4 mM of nitrogen are found to be best for degradation.

6.5.2 Phenylamide Compounds

Most of the herbicides used in agriculture belong to the phenylamide class of compounds and are based on substituted anilines. Phenylamide compounds can be subdivided into three categories: acylanilides, phenylureas, and phenylcarbamates. Kaufman and Blake (1973) described the microbial degradation of several acetamide, acylanilide, phenylurea, carbamate, and toluidine herbicides.

6.5.2.1 Acylanilides. Acylanilides include propanil, alachlor, butachlor, propachlor, metolachlor, karsil, dicryl, and others. Certain soil fungi have the ability to hydrolyze most acylanilide herbicides by acylamidase, resulting in the formation of anilines and an aliphatic group (Bartha and Pramer, 1970). Species of *Penicillium* and *Pullularia* and *Fusarium solani* utilize most of these herbicides as the sole sources of carbon and energy. Lanzilotta and Pramer (1970a,b) reported the release of 3,4-dichloroaniline (DCA) by hydrolysis during growth of *F. solani* on propanil. DCA and 3,3′,4,4′-tetrachloroazobenzene (TCAB) are also released in propanil-treated soil (Bartha and Pramer, 1970).

The first report of degradation of alachlor (2-chloro-*N*-methoxymethyl-*N*-(2,6-diethylphenyl)acetamide) to a number of ^{14}C polar and nonpolar metabolites by the white-rot fungi *Ceriporiopsis subvermispora* and *Phlebia tremellosa* was that of Ferrey et al. (1994). Alachlor was not recovered from the culture of *C. subvermispora* after 122 days, while 11% was collected from combined aqueous and wood extract fractions of the culture of *P. tremellosa.* Metabolites appear to be produced through different degradative pathways. Mineralization of alachlor was shown to be slow in agricultural soils, less than 3% in 30 days (Novick et al., 1986) and 120 days (Yen et al., 1994). *Cunninghamella elegans* transforms 98.6% of [^{14}C]alachlor, and hydroxylation of one of the aryl ethyl side chains is responsible for the metabolism (Pothuluri et al., 1993). Isomers of 2-chloro-*N*-(methoxymethyl)-*N*-(2-ethyl-6-(1-hydroxyethyl)-phenyl)acetamide and 2-chloro-*N*-(2,6-diethylphenyl)acetamide are identified as the major metabolic products, with 2-chloro-*N*-(methoxymethyl)-*N*-(2-vinyl-6-ethylphenyl)acetamide as the minor metabolic product. These hydroxylated products are considered less toxic than alachlor. Levanon (1993) reported alkyl side-chain cleavage of alachlor with minimum mineralization of the ring carbon due to fungal activity. The side chain of alachlor is utilized as a carbon source by microorganisms (Sun et al., 1990). *C. elegans* has the ability to metabolize metolachlor to hydroxylated products that are considered less toxic than the parent compound (Pothuluri et al., 1997). As of this date, no isolated microorganism is known to mineralize the aromatic ring of alachlor to CO_2.

F. solani degraded butachlor [2-chloro-2′,6′-diethyl-*N*-(butoxymethyl) acetanilide], resulting in the formation of at least 30 to 32 metabolites (Chakraborty and Bhattacharyya, 1991). Of these, three compounds, 2,6-diethylaniline (III), 2′,6′-diethylacetanilide (IV), and 2-chloro-2′,6′-diethylacetanilide (XII) were confirmed in *F. solani* and *F. oxysporum.* Nine other degradative products (II, V to XI, and XIII) could be identified only by GC-MS in *F. solani.* The structures of the remaining compounds were not determined. Possible pathways for the butachlor degradation by *F. solani* are shown in Figure 6.2. The main degradative pathways include dechlorination, dehydrogenation, debutoxymethylation, C-dealkylation, N-dealkylation, O-dealkylation, hydroxylation, and cyclization. *Mucor sufui* NTU-358 degrades butachlor, forming 8 to 12 metabolites (Chen and Wu, 1978). More than 10 metabolites have been found as a result of the metabolism of butachlor by *Chaetomium globosum* (Lee, 1978). Similar degradative pathways of α-chloroacetanilide herbicides have been revealed in bacteria and fungi. Propachlor can be detoxified by some species of *Aspergillus*, *Penicillium*, and *Trichoderma viride* (Rankov and Valev, 1977). McGahen and Tiedje (1978) studied the metabolism of antor and metolachlor using *Chaetomium globosum.*

6.5.2.2 *Phenylureas.* Phenylureas, one of the most prominent and diversified groups of herbicides, were discovered in the early 1950s. About 25
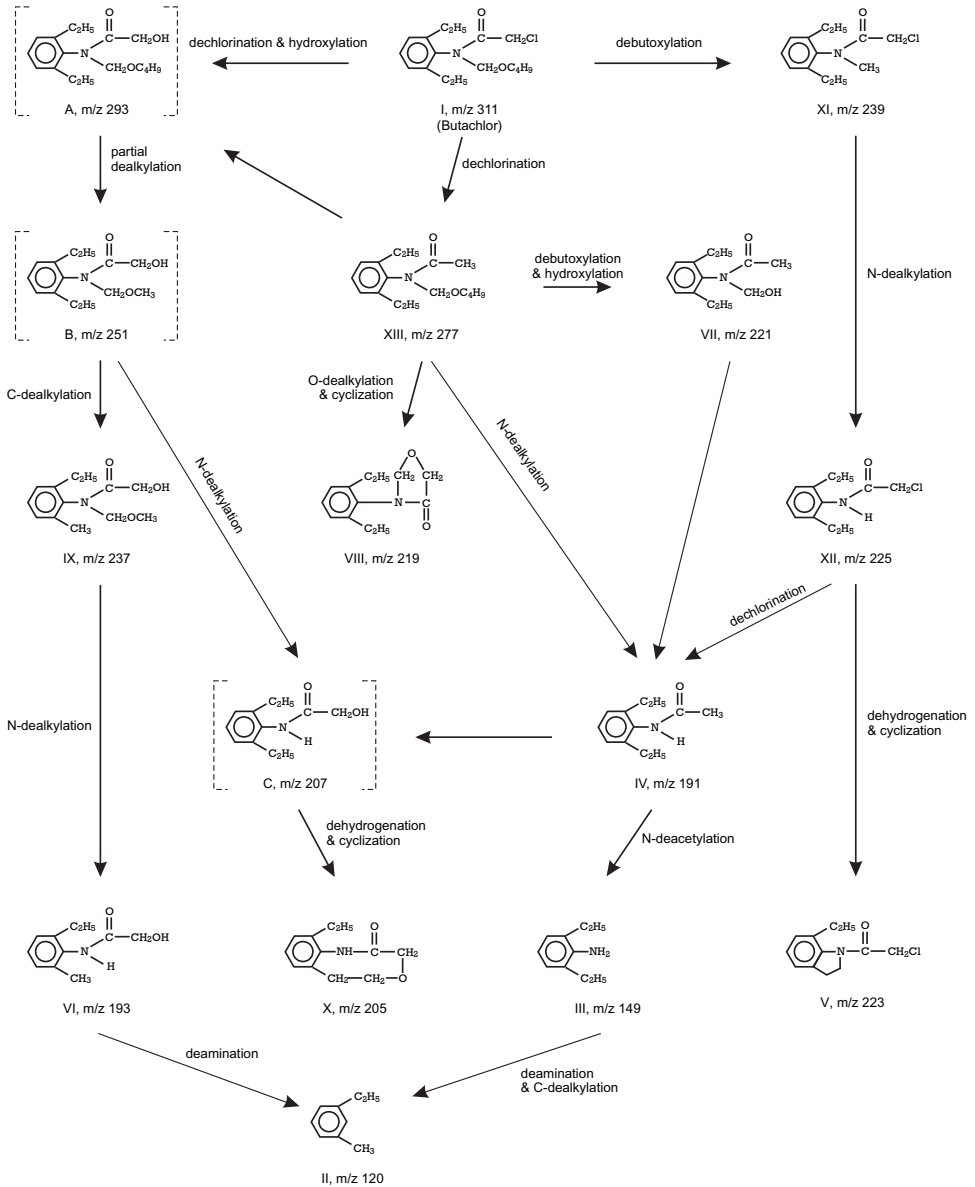
Figure 6.2 Partial pathways for the degradation of butachlor by *Fusarium solani*. [Reprinted from Chakraborty and Bhattacharyya (1991), copyright © with permission from Elsevier.]

herbicides of phenylurea origin were marketed. Major phenylureas of environmental concern are linuron, diuron, chlortoluron, and isoproturon, and of lesser environmental concern are monolinuron, monuron, and others. The important mechanisms of phenylurea detoxification are dealkylation, N-alkylation, N-methoxylation, or dimethylation. Linuron decomposes with the formation of metabolites by *Aspergillus niger*, *Geotrichum candidum*, *Trichoderma viride*, and two species of *Cladosporium* (Glad et al., 1981). Of 12 species, *Botrytis cinerea* depletes linuron almost completely, and *Sordaria superba* depletes it 25% in liquid media (Bordjiba et al., 2001).

Rhizoctonia solani has been shown to degrade diuron, chlorbromuron, and a wide variety of other substituted phenylureas (e.g., fenuron, fluometuron, linuron, metobromuron) (Weinberger and Bollag, 1972). Transformation of these herbicides occurs irrespective of variations in ring structures and functional groups. This broad substrate specificity behavior indicates that the fungus contains certain enzymes or enzymatic systems. Stepwise demethylation occurred during transformation of fluometuron by *R. solani*, with the formation of two major metabolites (Rickard and Camper, 1978). Oxidative dealkylation of diuron, monuron, and three other phenylureas by *Cunninghamella echinulata* has been shown to take place (Tillmanns et al., 1978).

About 4%, 7%, and 11% of the strains of soil fungi belonging to different taxonomic groups deplete about 50% each of chlortoluron, diuron, and isoproturon, respectively (Vroumsia et al., 1996). On GS medium, *R. solani* transformed chlortoluron completely after 15 days and diuron and isoproturon after 10 days. Constitutive and intracellular enzymes appear to be responsible for the degradation of three phenylureas. Of more than 20 species, *Mortierella hyalina* and *Mucor racemosus* degraded chlortoluron by 50% or more (Seigle-Murandi et al., 1991, 1992). Of 100 strains of Micromycetes, comprising Ascomycetes, Basidiomycetes, and yeasts, nearly 17, 4, and 2 strains depleted 50% of isoproturon, diuron, and chlortoluron, respectively (Khadrani et al., 1999). The best degraders were *Bjerkandera adusta* and *Oxysporus* sp. *B. adusta* produced MnP and LiP, and fungal biomass did not exhibit adsorption. Of five yeasts, *Cryptococcus albidus* degraded chlortoluron (23%), diuron (28%), and isoproturon (54%) efficiently. This indicates a potential to treat contaminated soils and wastewater and use in a bioremediation process. Of 31 species, *Botrytis cinerea* removed metobromuron from the medium almost completely (Bordjiba et al., 2001). *Coriolus versicolor* degraded diuron almost completely in liquid culture after 42 days (Bending et al., 2002). All other fungi showed less than 22% degradation. [^{14}C]Diuron ring residue analyses reveal less than 60% ring-C remaining in cultures of *Coriolus versicolor* and *Stereum hirsutum* and over 77% remaining in cultures of *Hypholoma fasciculare* and *Agrocybe semiorbicularis*. No correlation existed between the presumptive ligninolytic activity and the degradation of diuron.

6.5.2.3 *Phenylcarbamates.* Phenylcarbamates, including propham and chlorpropham, exhibit persistence in soils for a short duration due to

effectiveness at low applications. Phenylcarbamates are degraded by micro-organisms, including utilization of propham and chlorpropham, by several species of fungi (McClure, 1974). 2-Amino-4-chlorophenol has been detected in chlorpropham-treated soil using *Fusarium oxysporum* (Fletcher and Kaufman, 1979).

6.5.3   *s*-Triazine Compounds

Extensive use of chlorinated *s*-triazine compounds has contributed to con-tamination of surface water and groundwater in many countries. *s*-Triazine compounds are divided into two classes, such as chlorinated and sulfur-containing *s*-triazine compounds. *s*-Triazine chlorinated compounds are atrazine and simazine, and sulfur-containing compounds are ame-tryne, prometryne, and simetryne. Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine] is a widely used herbicide and is highly persistent in the environment, with a half-life of 300 to 500 days. An accept-able concentration of atrazine in water is less than $3\mu g/l$ (USEPA, 1991). Cook (1987) reviewed the biodegradation of *s*-triazines in wastewater by microorganisms. Alva and Singh (1990) described the sorption of four herbi-cides, including simazine, at various horizons at two soils.

Several soil fungi have been shown to release $^{14}CO_2$ from ring- and side-chain-labeled atrazine (Kaufman and Blake, 1970). *Phanerochaete chryso-sporium* mineralized 24% of atrazine after 16 days through the ethyl group (Mougin et al., 1994). More-polar hydroxylated and/or N-alkylated metabo-lites are produced during degradation. Further degradation of dealkylated metabolites does not occur; thus it accumulates in the medium. N-Dealkylation is more common than hydroxylation in fungi, resulting in direct or secondary metabolism leading to hydrolysis of atrazine.

Biobeds are on-farm bioremediation systems used to retain pesticides and facilitate natural attentuation. Fogg (2001) discussed uses of biobeds related to the behavior of pesticides in soils and water. Selected white-rot fungi can be used in on-farm pesticide bioremediation systems. *Hypholoma fasciculare* and *Stereum hirsutum* are the best degraders of terbuthylazine in liquid culture (Bending et al., 2002). *Coriolus versicolor* exhibited the highest ability to degrade atrazine in liquid media. *H. fasciculare* attained a high level of degradation of atrazine in the biobed matrix.

Pleurotus pulmonarius degrades atrazine in liquid culture and produces mainly N-dealkylated metabolites such as deethylatrazine (CAIT), deisopro-pylatrazine (CEAT), deethyldeisopropylatrazine (CAAT), and a hydroxy-propyl metabolite, hydroxyisopropylatrazine (CEIOT) (Masaphy et al., 1993). These metabolites constitute more than 75% of the disappearance of atrazine. Solid-state fermentation is suggested as a means to detoxify carbofuran and atrazine (Berry et al., 1993). *Pleurotus pulmonarius* supported the degrada-tion of atrazine during solid-state fermentation on a mixture of cotton and wheat straw (CWS) (Masaphy et al., 1996a). Atrazine does not mineralize in

6 weeks. Unlike degradation in liquid culture, atrazine is transformed into chlorinated and dechlorinated metabolites as a result of inoculated fungus and bacterial population. The dechlorinated metabolites include hydroxyatrazine (OEIT), *N*-isopropylammeline (OAIT), *N*-ethylammeline (OEAT), and ammeline (OAAT). Atrazine is detoxified in an organic matrix by both adsorption and biodegradation. Atrazine transformation by manganese also enhanced the whole-cell culture of *Pleurotus pulmonarius* (both as $MnCl_2$ and $MnSO_4$) at a concentration of up to 300 μM (Masaphy et al., 1996b). The N-dealkylated metabolites are accumulated to a larger extent than propylhydroxylated metabolites. The brown color of the mycelium, lipid peroxidation, oxygenase and peroxidating activities, the cytochrome P450 concentration, and interaction between atrazine and cell extract are increased. Antioxidants prove inhibitory either on atrazine transformation by the mycelium or on accumulation of metabolites. It seems that Mn(II) stimulates oxidative activity, which in turn is responsible for enhancing the biotransformation of atrazine.

Metamitron is a member of the 1,2,4-triazinone herbicides. Of 21 species, only *Alternaria solani*, *Drechslera australiensis*, and *Absidia fusca* reduce 10 to 16% of metamitron in the medium (Bordjiba et al., 2001). *Cunninghamella echinulata* and *Rhizopus japonicus* degrade metamitron to desaminometamitron by desamination (Engelhardt and Wallnofer, 1978). *Botrytis cinerea*, *Sordaria superba*, and *A. fusca* degraded 61%, 64%, and 95% of metribuzin [4-amino-6-*t*-butyl-3-methylthio-1,2,4-triazin-5(4H)-one] after 5 days, respectively (Bordjiba et al., 2001). *C. echinulata* and *R. japonicus* can completely transform metribuzin after 4 weeks (Schilling et al., 1985). During degradation of metribuzin, *C. echinulata* produces two metabolites, desaminometribuzin and desaminodiketometribuzin. Other species of fungi (i.e., *Aspergillus niger*, *Penicillium lilacinum*, and *Fusarium oxysporum*) showed 27 to 45% degradation of metribuzin.

6.5.4 Miscellaneous Compounds

Miscellaneous compounds include herbicides of lesser or minor environmental concern. The degradation of dinitroanilines, comprising trifluralin, nitralin, dinitramine, benefin, and so on, and thiocarbamates, comprising molinate and diallate, has been described (Singh et al., 1991). Transformation of trifluralin by fungi, fungal metabolites, and degradative pathways have been depicted by Zayed et al. (1983). *F. oxysporum* is one of the main fungal isolates capable of the degradation of carbamate (Lee, 1984). Savin and Amador (1998) reported on microbial degradation of norflurazon in a bog soil.

6.6 FUNGAL DEGRADATION OF FUNGICIDES

Fungicides are used against various types of plant diseases caused by fungi. They were applied rigorously against plant pathogens in the 1950s and 1960s.

In recent years, the use of fungicides has declined considerably due to the high toxicity and accumulative nature in the environment. Table 6.3 notes the rates of degradation and mineralization and metabolic products of certain fungicides by fungi.

6.6.1   Organomercurial Compounds

Organomercurial compounds include semesan, panodrench, and panogen and were used to control plant diseases since the beginning of the twentieth century. Their use was stopped several decades ago, due to their high toxicity, so they are of minor environmental concern. Semesan is rendered inactive by inoculation with *Penicillium* and *Trichoderma viride* (Spanis and Munnecke, 1962; Spanis et al., 1962).

6.6.2   Organosulfur Compounds

Organosulfur compounds, include the dithiocarbamates, which enjoyed popularity globally in the 1960s. Two categories of dithiocarbamates are recognized, monoalkyl and dialkyldithiocarbamates. Monoalkyldithiocarbamates include maneb, zineb, and mancozeb, and dialkyldithiocarbamates include ferbam, thiram, and ziram. Thiram is detoxified in soil by *Pythium ultimatum* (Munnecke and Mickail, 1967) and *Rhizoctonia solani* (Chatrath and Raju, 1986). Of three white-rot fungi, *Stereum hirsutum* performs best on iprodione, a dicarboximide, on a biobed matrix (Bending et al., 2002). Hydroxylation of *trans*-2-fluorocycloalkyl *N*-phenylcarbamates with *Beauveria bassiana* occurs at the 4-position, resulting in a mixture of diastereomeric products (Haufe et al., 2002). In one study, about 93% the dicarboximide fungicide vinclozolin   [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione] was transformed after 96 hours into four metabolites by *Cunninghamella elegans* (Pothuluri et al., 2000). Biotransformation occurred on the oxazolidine-2,4-dione portion of vinclozolin. The metabolites identified were the 3R and 3S isomers of 3′,5′-dichloro-2,3,4-trihydroxy-2-methylbutyranilide (II,   III),   *N*-(2-hydroxy-2-methyl-1-oxobuten-3-yl)-3,5-dichlorophenyl-1-carbamic acid (I), and 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (IV). A scheme of transformation of vinclozolin either by hydration and/or decarboxylation is shown in Figure 6.3. Vinclozolin is metabolized by the addition of water to the ester group to form metabolite I. This is a major metabolite and is accounted for 50% of the total metabolism. Decarboxylation of the oxazolidine portion of vinclozolin resulted in the formation of metabolite IV. Metabolites II and III were formed by an epoxide hydrolase reaction via ethylene dihydroxylation of metabolite (IV). These two novel metabolites accounted for 33% of the total metabolism at 168 hours of incubation. Thus, biotransformation of vinclozolin by *C. elegans* took place via multiple-site oxidation.

TABLE 6.3 Fungal Degradation of Fungicides

Fungus	Fungicide Conc.	Medium	Degradation/ Mineralization Rate (%)	Metabolic Products	Duration (days)	Reference
Coriolus versicolor	Iprodione, 20μg/g	Biobed matrix	58		42	Bending et al., 2002
Hypholoma fasciculare			47			
Stereum hirsutum			63			
Cunninghamella elegans	Phenyl-[U-(ring)-^{14}C] Vinclozolin, 10 mg	Sabouraud dextrose broth	93	Four metabolites	4	Pothuluri et al., 2000

Vinclozolin

Esterase

N-(2-hydroxy-2-methyl-1-oxobuten-3-yl)-3,5-dichlorophenyl-1-carbamic acid (I)

Decarboxylation

3', 5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide (IV)

Epoxide Hydrolase H_2O

3R- & 3S- isomers of 3', 5'-dichloro-2, 3, 4-trihydroxy-2-methylbutyranilide (II, III)
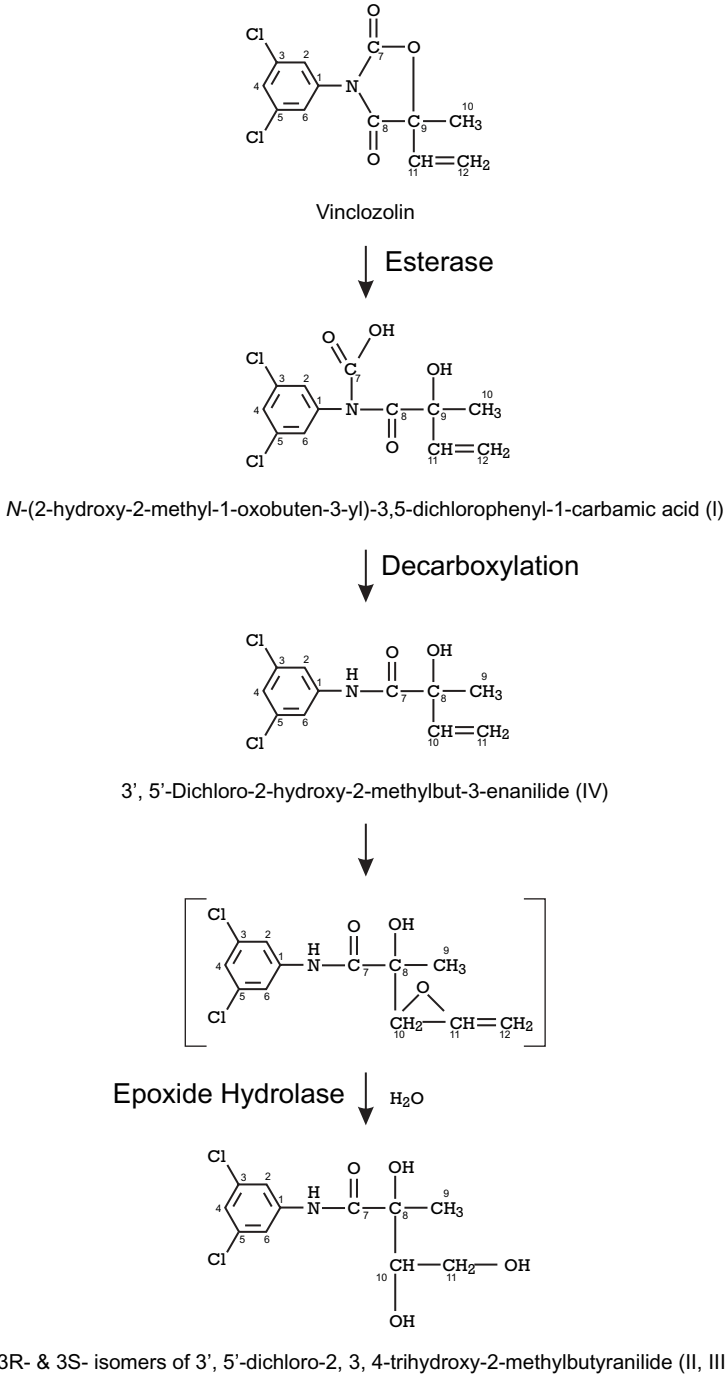
Figure 6.3   Biotransformation of vinclozolin by *Cunninghamella elegans*. The structure of the compound in parentheses is a proposed uncharacterized metabolite. [Reprinted from Pothuluri et al. (2000), copyright © with permission from American Chemical Society.]

6.6.3 Organophosphorus Compounds

Organophosphorus compounds include pyrazophos, kitazin P, edifenphos (hinosan), and inezin. *Pyricularia oryzae* metabolizes pyrazophos into certain compounds (de Waard, 1974). The mycelium of *P. oryzae* transformed edifenphos to *ortho*-phosphoric acid in a study by Uesugi and Tomizawa (1971a). A plausible degradation pathway of edifenphos, including des-*S*-phenylation, was also depicted. A similar degradation pathway for inezin by *P. oryzae* has been reported (Uesugi and Tomizawa, 1971b). In inezin, hydroxylation occurs at the *meta*-position rather than at the *para*-position as in edifenphos degradation by *P. oryzae*.

6.6.4 Aromatic and Heterocyclic Compounds

Aromatic compounds include quintozene [pentachloronitrobenzene (PCNB)] and chloroneb. Heterocyclic compounds include classes of systemic fungicides, such as benzimidazoles (benomyl, carbendazim), carboxylic acid anilides (carboxin and mebenil), and triazolylmethane (triadimefon). Degradation of PCNB by several fungi and actinomycetes in pure culture has been known for four decades (Chacko et al., 1966; Nakanishi and Oku, 1969). *Rhizoctonia solani* demethylates chloroneb to a nontoxic metabolic product (Hock and Sisler, 1969). *Coriolus versicolor* and *Stereum hirsutum* degraded 40% and 54% of a phenylamide fungicide and metalaxyl, respectively, on a biobed matrix after 42 days (Bending et al., 2002).

6.7 BIOTRANSFORMATION OF PESTICIDES BY FUNGAL ENZYMES

In plants and soils, isoxaflutole (5-cyclopropyl-1,2-oxazol-4-yl α,α,α-trifluoro-2-mesyl-*p*-tolyl ketone) is converted rapidly to its diketonitrile derivative (DKN), the active principle of herbicide. The diketonitrile derivative undergoes cleavage to an inactive benzoic acid analog (BZA). Transformation of pesticides by fungal enzymes is noted in Table 6.4. *Phanerochaete chrysosporium* and *Trametes versicolor* have the ability to transform diketonitrile to acid in liquid media (Mougin et al., 2000). In addition to BZA, another polar metabolite (M_2), produced during the same period, represents <3.7% of the amount of the parent compound. Diketonitrile degradation occurs at the end of the growing phase and appears to coincide with the production of extracellular laccases by both fungi. Purified laccase from *T. versicolor* in the presence of 1 mM 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) transforms diketonitrile with the formation of three metabolites, thus acting as a redox mediator. One of the metabolites is identified as BZA, a second shows the same retention time as that of compound M_2, and the third is a still more polar compound. However, laccase in the

TABLE 6.4 Biotransformation of Pesticides by Fungal Enzymes

Fungus/ Enzyme	Pesticide Conc.	Medium/ Bioreactor	Degradation/ Mineralization Rate (%)	Metabolic Products	Duration (minutes/ hours/days)	Reference
Phanerochaete chrysosporium	Diketonitrile, 30µM	Liquid medium	66	BZA (24.6%), unidentified metabolite	15d	Mougin et al., 2000
Trametes versicolor			60	BZA (15.1%), unidentified metabolite		
T. versicolor (laccase/ 1mM ABTS)	Diketonitrile, 10µM	Phosphate– citrate buffer		BZA, two unidentified polar metabolites	1h	Jolivalt et al., 2000
T. versicolor (laccase 220U)	2-HF, 0.1g/l	Immobilized onto a membrane	100	Insoluble products	5h	
P. chrysosporium	Methoxychlor, 10mM	Malonate buffer 10nkat MnP/ 0.1% Tween	65	Methoxychlor olefin (MCO), 4,4'-dimethoxybenzophenone (DMB)	24h	Hirai et al., 2004
T. versicolor (10nkat laccase/ 0.2mM HBT)		Malonate buffer	23	MCO, DMB		
P. chrysosporium (10nkat LiP)		Succinate buffer	28			
Bjerkandera adusta (6nM–2µM VP)	Dichlorophen; Bromoxynil, 30–50µM	Sodium acetate buffer, pH range 2–7		4-Chlorophenol-2,2'-methylenequinone; Dimer and trimer	10m	Davila-Vazquez et al., 2005
Fusarium oxysporum f. sp. *pisi* (10mg/l cutinase)	Malathion 10mg/l	10mM Tris–HCl buffer, pH 8.0	93	Malathion monoacid (MMA), malathion diacid (MDA)	48h	Kim et al., 2005
Candida cylindracea (100mg/l esterase)	100mg/l		94	Malathion monoacid (MMA)		

presence of 2 mM ABTS at acidic pH 3 supports the cleavage of DKN at the higher rates of 0.3 to 0.4 nM/h per unit.

Laccase from *T. versicolor* is immobilized onto a hydrophilic PVDF microfiltration membrane and is used for the removal of a herbicide derivative, N',N'-(dimethyl)-N-(2-hydroxyphenyl)urea (2-HF) from wastewater (Jolivalt et al., 2000). Laccase can oxidize 2-HF into insoluble products, which are separated by crossflow filtration through a microfiltration module. Laccase (220 U activity)-grafted membrane with a 60-cm^2 effective filtration area transforms 200 ml of a 2-HF solution of 0.1 g/l within less than 5 minutes. Most of the transformation products are insoluble and are retained by the membrane. However, HPLC measurement of pink permeates indicates that they are a small and soluble part of the oxidation products.

Manganese peroxidase (MnP) from *P. chrysosporium* ME-446 in the presence of Tween 80 dechlorinated 65% methoxychlor oxidatively, resulting in the formation of methoxychlor olefin (MCO) and 4,4′-dimethoxybenzophenone (DMB), in a study by Hirai et al. (2004). Laccase from *T. versicolor* IFO-6482 in the presence of 1-hydroxybenzotriazole (HBT) reduced methoxychlor by 23%, producing the same metabolites. Lignin peroxidase (LiP) from *P. chrysosporium* reduced methoxychlor by 28% without the formation of metabolites. MnP-Tween 80 and laccase-HBT degraded the MCO levels by 15% and 5% after 24 hours, respectively. LiP did not degrade the MCO. A possible mechanism for the dechlorination of methoxychlor by MnP-Tween or laccase-HBT treatment is shown in Figure 6.4. Mechanisms are also proposed for the oxidative dehalogenation of dichlorophen and polymerization of bromoxynil catalyzed by versatile peroxidase (VP) from *Bjerkandera adusta* (Davila-Vazquez et al., 2005). Malathion monoacid (MMA) and malathion diacid (MDA) are produced as a result of ester hydrolysis of malathion by cutinase of *Fusarium oxysporum* f. sp. *pisi* (Kim et al., 2005). However, MMA is the major degradation product of malathion detoxification by esterase of *Candida cylindracea*.

6.8 GENETIC MANIPULATION

Fungi can degrade a wide variety of pesticides under laboratory conditions. Methods to decontaminate the environment from residues of pesticides are yet to be developed. Genes for the degradation of pesticides are discovered on plasmids, transposons, and/or chromosomes in bacteria. Plausible degradative pathways and organization of catabolic genes can lead to the development of bioengineered microorganisms to decontaminate the environment. This can be accomplished by enhancing the degradative capabilities of existing metabolic pathways either by inserting specific genes or by modifying the specificity of existing catabolic genes or additional enzymes from other microorganisms. Pesticide-degrading genes have been identified, isolated, and cloned in other organisms. A new recombinant strain is constructed by
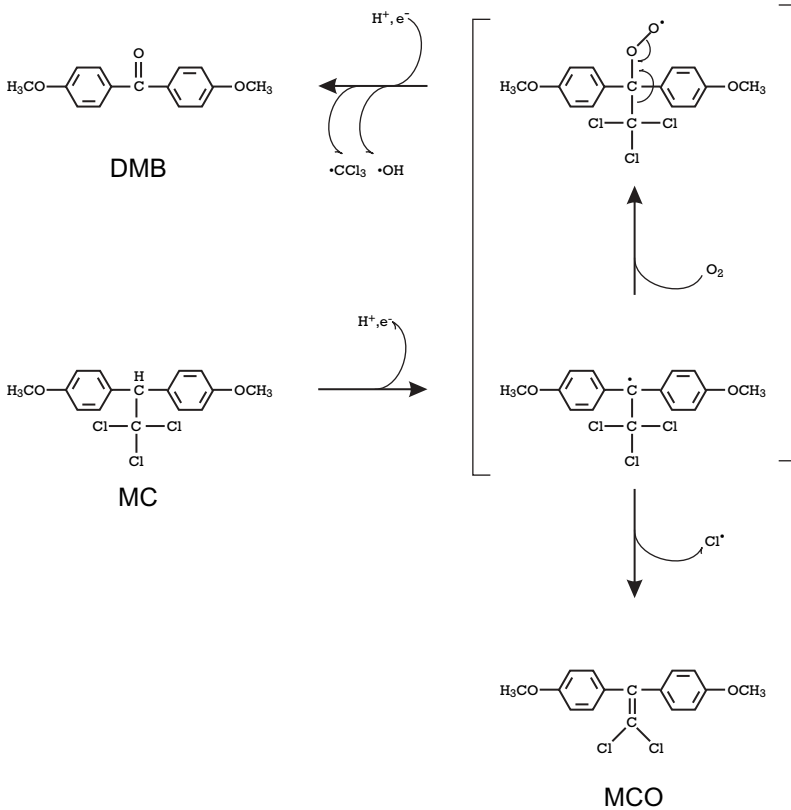
Figure 6.4 Tentative mechanism for dechlorination of methoxychlor by MnP-Tween 80 or laccase-HBT treatment using white-rot fungi. DMB, 4,4′-dimethoxybenzophenone; MC, methoxychlor; MCO, methoxychlor olefin. [Reprinted from Hirai et al. (2004), copyright © with permission from Elsevier.]

transferring a 2,4-D-degradative plasmid, pJP4, from *Alcaligenes eutrophus* JMP134 to the 2,4,5-T-degrading *Pseudomonas cepacia* AC1100 (Haugland et al., 1990). This new bioengineered strain, designated RHJ1, has been shown to degrade 2,4-D and 2,4,5-T simultaneously in liquid medium. Microbial adaptation to pesticides and the molecular aspects of pesticide degradation by microorganisms have been well elucidated (Kearney and Kellog, 1985; Kumar et al., 1996).

Unlike bacteria, the role of biotechnological innovations related to degradation of pesticides by fungi is relatively less well understood. Moreover, bacteria and fungi exhibit different mechanisms in the biodegradation of pesticides. Gene amplification for lignin peroxidase is highly significant because of its involvement in the degradation of DDT, lindane, and other pesticides. This can open doors for cloning more genes specific for the degradation of pesticides. Furthermore, protoplast fusion of cells in specific fungi

needs to be explored to amplify the degradative abilities related to pesticides.

6.9 CONCLUSIONS AND FUTURE PERSPECTIVES

Fungi have revealed a remarkable ability to degrade a wide array of pesticides. The knowledge described above focused on the characterization of degradation and mineralization of pesticides by fungi. Metabolites were identified in a number of cases, and degradative pathways were described in some cases. Certain fungi and bacteria exhibit similar types of biochemical reactions. Certain biochemical reactions, enzymes, transformations, and pathways are noteworthy exclusively in fungi. Presently, little is known on the isolation of specific fungi with the unique ability to mineralize pesticides from the natural environment, and continuous efforts are required in this direction.

The current state of knowledge is derived primarily from cultural studies on individual microorganisms degrading individual pesticides, especially bacteria and to a lesser extent, fungi and actinomycetes. However, microorganisms and pesticides do not exist in the individual state in complex environments. In soils, pesticides are always attacked simultaneously by a group of microorganisms with their enzymes. Sometimes, degradation of a pesticide by a fungus leads to the formation of metabolic products, and further degradation is not possible, and these products are broken down by another fungus or other microorganism(s) in the soil, thus leading to partial or complete transformation. On the other hand, microorganisms degrade different types of pesticides present in different concentrations in the soil. This complex and heterogeneous environment of pesticides and microorganisms leads to synergistic or antagonistic interactions among them. The evidence suggests the removal of HMN from the soil by the combined action of photolysis at the surface and degradation by soil microbes and *Phanerochaete chrysosporium* (Abernathy and Walker, 1993). A higher rate of simultaneous mineralization of 2,4-D and 2,4,5-T by *P. chrysosporium* is achieved than when they are each tested alone (Yadav and Reddy, 1993). More research is required to elucidate these interactions and the mode of action of fungal enzymes in pesticide transformations. Such knowledge will lead to a better understanding of real situations in the environment.

Adding suitable bacteria or fungi through soil enrichment techniques has not resulted in much mineralization of pesticides. The long-term goal is to find promising fungal species and reliable application methods to introduce into rhizospheres for enhanced degradation of pesticides. *Trichoderma harzianum* appears to be an excellent candidate for this purpose. This species can thrive in various rhizospheres as a biocontroller against several plant pathogenic microbes; it has the advantage of tolerating fungicides such as benomyl, and the feasibility of a biomarker; it exhibits the ability to degrade

cellulose, which shows the highest competitiveness in rhizosphere. The role of *Bjerkandera adusta* in contaminated soils requires confirmation of its use in practical treatment systems (Khadrani et al., 1999). Due to the amazing versatility of biological systems, a possibility exists for the commercial use of fungi for the bioremediation of pesticides.

REFERENCES

Abernathy, G.A., and J.R.I. Walker (1993) Degradation of the insecticide hydramethylnon by *Phanerochaete chrysosporium. Biodegradation* **4**: 131–139.

Alexander, M.Y. (1990) Amperometric biosensors. In: *Bioinstrumentation Research Developments and Application*s, D.L. Wise, ed. Butterworth-Heinemann, Boston, pp. 1317–1349.

Al-Mihanna, A.A., A.K. Salama, and M.Y. Abdalla (1998) Biodegradation of chlorpyrifos by either single or combined cultures of some soilborne plant pathogenic fungi. *J. Environ. Sci. Health B* **33**: 693–704.

Alva, A.K., and M. Singh (1990) Sorption of bromacil, diuron, norflurazon, and simazine at various horizons in two soils. *Bull. Environ. Contam. Toxicol.* **45**: 365–374.

Anderson, J.P.E., and E.P. Lichtenstein (1971) Effect of nutritional factors on DDT degradation by *Mucor alternans. Can. J. Microbiol.* **17**: 1291–1298.

Anon. (1996) News update. *Biosens. Bioelectron.* **10**: iv.

Anon. (1998) *Pesticides 1998*. HMSO for the Pesticides Safety Directorate and Health and Safety Executive, London.

Baarschers, W.H., and H.S. Heitland (1986) Biodegradation of fenitrothion and fenitrooxon by the fungus *Trichoderma viride. J. Agric. Food Chem.* **34**: 707–709.

Bartha, R., and D. Pramer (1970) Metabolism of acylanilide herbicides. *Adv. Appl. Microbiol.* **13**: 317–341.

Bending, G.D., M. Friloux, and A. Walker (2002) Degradation of contrasting pesticides by white rot fungi and its relationship with ligninolytic potential. *FEMS Microbiol. Lett.* **212**: 59–63.

Berry, D.F., R.A. Tomkinson, G.H. Hetzel, D.E. Mullins, and R.W. Young (1993) Evaluation of solid-state fermentation techniques to dispose of atrazine and carbofuran. *J. Environ. Qual.* **22**: 366–374.

Besombes, J.-J., S. Cosnier, P. Labbe, and G. Reverdy (1995) A biosensor as warning device for the detection of cyanide, chlorophenols, atrazine and carbamate pesticides. *Anal. Chim. Acta* **311**: 255–263.

Bollag, J.M. (1974) Microbial transformation of pesticides. *Adv. Appl. Microbiol.* **18**: 75–130.

Bordjiba, O., R. Steiman, M. Kadri, A. Semadi, and P. Guiraud (2001) Removal of herbicides from liquid media by fungi isolated from a contaminated soil. *J. Environ. Qual.* **30**: 418–426.

Bumpus, J.A., and S.D. Aust (1987) Biodegradation of DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane] by the white rot fungus *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **53**: 2001–2008.

Bumpus, J.A., R.H. Powers, and T. Sun (1993a) Biodegradation of DDE (1,1-dichloro-2,2-bis(4-chlorophenyl)ethene) by *Phanerochaete chrysosporium. Mycol. Res.* **97**: 85–98.

Bumpus, J.A., S.N. Kakar, and R.D. Coleman (1993b) Fungal degradation of organophosphorus insecticides. *Appl. Biochem. Biotechnol.* **39–40**: 715–726.

Chacko, C.I., J.L. Lockwood, and M. Zabik (1966) Chlorinated hydrocarbon pesticides: degradation by microbes. *Science* **154**: 893–895.

Chakraborty, S.K., and A. Bhattacharyya (1991) Degradation of butachlor by two soil fungi. *Chemosphere.* **23**: 99–105.

Chatrath, M.S., and T.N. Raju (1986) Persistence of ^{35}S labeled thiram in soil. *Indian Phytopathol.* **39**: 312–313.

Chen, Y.L., and T.C. Wu (1978) Degradation of herbicide butachlor by soil microbes. *J. Pestic. Sci.* **3**: 411–417.

Cook, A.M. (1987) Biodegradation of *s*-triazine xenobiotics. *FEMS Microbiol. Rev.* **46**: 93–116.

Davila-Vazquez, G., R. Tinoco, M.A. Pickard, and R. Vazquez-Duhalt (2005) Transformation of halogenated pesticides by versatile peroxidase from *Bjerkandera adusta. Enzyme Microb. Technol.* **36**: 223–231.

de Waard, M.A. (1974) Mechanism of action of the organophosphorus fungicide pyrazophos. *Meded. Landouwhogesch. Wagening.* **74–14**: 1–97.

Dzantiev, B.B., A.V. Zherdev, M.F. Yulaev, R.A. Sitdikov, N.M. Dimitrieva, and I.Y. Moreva (1996) Electrochemical immunosensors for determination of the pesticides 2,4-dichlorophenoxy acetic acid and 2,4,5-trichlorophenoxy acetic acid. *Biosens. Bioelectron.* **11**: 179–185.

El-Zorgani, G.A., and M.E.H. Omer (1974) Metabolism of endosulfan isomers by *Aspergillus niger. Bull. Environ. Contam. Toxicol.* **12**: 182–185.

Engelhardt, G., and P.R. Wallnofer (1978) Microbial transformation of triazinone herbicide metamitron to desaminometamitron. *Chemosphere* **7**: 463–466.

Engst, R., and M. Kujawa (1968) Enzymatischer Addau des DDT durch Schimmelpilze. 3. Mitt. Darstellung des 2,2-bis(*p*-chlorophenyl)-acetaldehyds (DDHO) and seine Bedeutung im Abbaucyclus. *Nahrung* **12**: 783–785.

Fernando, T., S.S. Aust, and J.A. Bumpus (1989) Effects of culture parameters on DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane] by *Phanerochaete chrysosporium. Chemosphere* **19**: 1387–1398.

Ferrey, M.L., W.C. Koskinen, R.A. Blanchette, and T.A. Burnes (1994) Mineralization of alachlor by lignin-degrading fungi. *Can. J. Microbiol.* **40**: 795–798.

Fletcher, C.L., and D.D. Kaufman (1979) Hydroxylation of monochloroaniline pesticide residues by *Fusarium oxysporum* Schlecht. *J. Agric. Food Chem.* **27**: 1127–1130.

Fogg, P. (2001) Biobeds: safe disposal of pesticide waste and washings. In: *Pesticide Behaviour in Soils and Water*, British Crop Protection Council Symposium 78, A. Walker, ed. British Crop Protection Council, Surrey, UK, pp. 217–222.

Foght, J., T. April, K. Biggar, and J. Aislabie (2001) Bioremediation of DDT-contaminated soils: a review. *Bioremed. J.* **5**: 225–246.

Gizeli, E., and C.R. Lowe (1996) Immunosensors. *Curr. Opin. Biotechnol.* **7**: 66–71.

Glad, G., B. Goransson, T. Popoff, O. Theander, and N.T.L. Thorstensson (1981) Decomposition of linuron by fungi isolated from soil. *Swed. J. Agric. Res.* **11**: 127–134.

Grifoll, M., and K.E. Hammel (1997) Initial steps in the degradation of methoxychlor by the white rot fungus *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **63**: 1175–1177.

Haufe, G., D. Wolker, and R. Frohlich (2002) Selectivity of biohydroxylation with *Beauveria bassiana* of *trans*-2-fluorocycloalkyl *N*-phenylcarbamates. *J. Org. Chem.* **67**: 3022–3028.

Haugland, R.A., D.J. Schlemm, R.P. Lyons III, P.R. Sferra, and A.M. Chakrabarty (1990) Degradation of the chlorinated phenoxyacetate herbicides 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid by pure and mixed bacterial cultures. *Appl. Environ. Microbiol.* **56**: 1357–1362.

Hirai, H., S. Nakanishi, and T. Nishida (2004) Oxidative dechlorination of methoxychlor by ligninolytic enzymes from white-rot fungi. *Chemosphere* **55**: 641–645.

Hock, W.K., and H.D. Sisler (1969) Metabolism of chloroneb by *Rhizoctonia solani* and other fungi. *J. Agric. Food Chem.* **17**: 123–128.

Jolivalt, C., S. Brenon, E. Caminade, C. Mougin, and M. Pontie (2000) Immobilization of laccase from *Trametes versicolor* on a modified PVDF microfiltration membrane: characterization of the grafted support and application in removing a phenylurea pesticide in wastewater. *J. Membr. Sci.* **180**: 103–113.

Katayama, A., and F. Matsumura (1993) Degradation of organochlorine pesticides, particularly endosulfan, by *Trichoderma harzianum. Environ. Toxicol. Chem.* **12**: 1059–1065.

Kaufman, D.D., and J. Blake (1970) Degradation of atrazine by soil fungi. *Soil Biol. Biochem.* **2**: 73–80.

Kaufman, D.D., and J. Blake (1973) Microbial degradation of several acetamide, acylanilide, carbamate, toluidine and urea pesticides. *Soil Biol. Biochem.* **5**: 297–308.

Kearney, P.C., and S.T. Kellog (1985) Microbial adaptation to pesticides. *Pure Appl. Chem.* **57**: 389–403.

Kennedy, D.W., S.D. Aust, and J.A. Bumpus (1990) Comparative biodegradation of alkyl halide insecticides by the white rot fungus, *Phanerochaete chrysosporium* (BKM-F-1767). *Appl. Environ. Microbiol.* **56**: 2347–2353.

Khadrani, A., F. Seigle-Murandi, R. Steiman, and T. Vroumsia (1999) Degradation of three phenylurea herbicides (chlortoluron, isoproturon and diuron) by micromycetes isolated from soil. *Chemosphere* **38**: 3041–3050.

Khavkin, M.J., and J.A. Khavkin (1996) A ferromagnetic biosensor for simple assay of OP pesticides. *Anal. Lett.* **29**: 1041–1054.

Khindaria, A., I. Yamazaki, and S.D. Aust (1995) Veratryl alcohol oxidation by lignin peroxidase. *Biochemistry* **34**: 16860–16869.

Kim, Y.-H., J.-Y. Ahn, S.-H. Moon, and J. Lee (2005) Biodegradation and detoxification of organophosphate insecticide, malathion by *Fusarium oxysporum* f. sp. *pisi* cutinase. *Chemosphere* **60**: 1349–1355.

Kramer, P.M. (1996) Biosensors for measuring pesticide residues in the environment: past, present and future. *J. AOAC Int.* **79**: 1245–1254.

Kullman, S.W., and F. Matsumura (1996) Metabolic pathways utilized by *Phanero-chaete chrysosporium* for degradation of the cyclodiene pesticide endosulfan. *Appl. Environ. Microbiol.* **62**: 593–600.

Kumar, S., K.G. Mukerji, and R. Lal (1996) Molecular aspects of pesticide degradation by microorganisms. *Crit. Rev. Microbiol.* **22**: 1–26.

Lanzilotta, R.P., and D. Pramer (1970a) Herbicide transformation. I. Studies with whole cells of *Fusarium solani. Appl. Microbiol.* **19**: 301–306.

Lanzilotta, R.P., and D. Pramer (1970b) Herbicide transformation. I. Studies with an acylamidase of *Fusarium solani. Appl. Microbiol.* **19**: 307–313.

Lee, J.K. (1978) A study on degradation of butachlor by a soil fungus *Chaetomium globosum. J. Korean Agric. Chem. Soc.* **21**: 1–10.

Lee, A. (1984) EPTC (*s*-ethyl *N*,*N*-dipropylthiocarbamate)-degrading microorganisms isolated from a soil previously exposed to EPTC. *Soil Biol. Biochem.* **16**: 529–531.

Lestan, D., M. Lestan, J.A. Chapelle, and R.T. Lamar (1996) Biological potential of fungal inocula for bioaugmentation of contaminated soils. *J. Ind. Microbiol.* **16**: 286–294.

Levanon, D. (1993) Roles of fungi and bacteria in the mineralization of the pesticides atrazine, alachlor, malathion and carbofuran in soil. *Soil Biol. Biochem.* **25**: 1097–1105.

Lewis, D.L., D.F. Paris, and G.L. Baughman (1975) Transformation of malathion by a fungus, *Aspergillus oryzae* isolated from a freshwater pond. *Bull. Environ. Contam. Toxicol.* **13**: 596–601.

Maloney, S.E. (2001) Pesticide degradation. In: *Fungi in Bioremediation*, G.M. Gadd, ed. Cambridge University Press, Cambridge, pp. 188–223.

Martens, R. (1972) Der Abbau von Endosulfan durch Mikrooganismen des Bodens. *Schriftenr. Ver. Wasser-Boden-Lufthyg.* **37**: 167–173.

Martens, R. (1976) Degradation of [8,9-^{14}C]endosulfan by soil microorganisms. *Appl. Environ. Microbiol.* **31**: 853–858.

Masaphy, S., D. Levanon, J. Vaya, and Y. Henis (1993) Isolation and characterization of a novel atrazine metabolite produced by the fungus *Pleurotus pulmonarius*, 2-chloro-4-ethylamino-6-(1-hydroxyisopropyl)amino-1,3,5-triazine. *Appl. Environ. Microbiol.* **59**: 4342–4346.

Masaphy, S., D. Levanon, and Y. Henis (1996a) Degradation of atrazine by the lignocellulolytic fungus *Pleurotus pulmonarius* during solid-state fermentation. *Bioresour. Technol.* **56**: 207–214.

Masaphy, S., Y. Henis, and D. Levanon (1996b) Manganese-enhanced biotransformation of atrazine by the white rot fungus *Pleurotus pulmonarius* and its correlation with oxidation activity. *Appl. Environ. Microbiol.* **62**: 3587–3593.

Matsumura, F., and G.M. Boush (1968) Degradation of insecticides by a soil fungus *Trichoderma viride. J. Econ. Entomol.* **61**: 610–612.

McClure, G.W. (1974) Degradation of aniline herbicides by propham-adapted microorganisms. *Weed Sci.* **22**: 323–329.

McGahen, L.L., and J.M. Tiedje (1978) Metabolism of two new acylanilide herbicides, Antor herbicide (H-22234) and Dual (metalachlor) by the soil fungus *Chaetomium globosum. J. Agric. Food Chem.* **26**: 414–419.

Mougin, C., C. Laugero, M. Asther, J. Dubroca, P. Frasse, and M. Asther (1994) Biotransformation of the herbicide atrazine by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **60**: 705–708.

Mougin, C., C. Pericaud, C. Malosse, C. Laugero, and M. Asther (1996) Biotransformation of the insecticide lindane by the white rot basidiomycete *Phanerochaete chrysosporium*. *Pestic. Sci.* **47**: 51–59.

Mougin, C., F.-D. Boyer, E. Caminade, and R. Rama (2000) Cleavage of the diketonitrile derivative of the herbicide isoxaflutole by extracellular fungal oxidases. *J. Agric. Food Chem.* **48**: 4529–4534.

Munnecke, D.E., and K.Y. Mickail (1967) Thiram peristence in soil and control of damping off caused by *Pythium ultimatum*. *Phytopathology* **57**: 969–974.

Nakanishi, T., and H. Oku (1969) Metabolism and accumulation of PCNB by phytopathogenic fungi in relation to selective toxicity. *Phytopathology* **59**: 1761–1762.

Nikolelis, D., and C.C. Siontorou (1996) Flow-injection monitoring and analysis of mixtures of simazine, atrazine and propazine using filter-supported bilayer lipid membranes (BLMS). *Electroanalysis* **8**: 907–912.

Novick, N.J., R. Mukherjee, and M. Alexander (1986) Metabolism of alachlor and propachlor in suspensions of pretreated soils and in samples from groundwater aquifers. *J. Agric. Food Chem.* **34**: 721–725.

Pogacnik, L., and M. Franko (1999) Determination of organophosphate and carbamate pesticides in spiked samples of tap water and fruit juices by a biosensor with photothermal detection. *Biosens. Bioelectron.* **14**: 569–578.

Pothuluri, J.V., J.P. Freeman, F.E. Evans, T.B. Moorman, and C.E. Cerniglia (1993) Metabolism of alachlor by the fungus *Cunninghamella elegans*. *J. Agric. Food Chem.* **41**: 483–488.

Pothuluri, J.V., F.E. Evans, D.P. Doerge, M.I. Churchwell, and C.E. Cerniglia (1997) Metabolism of metolachlor by the fungus *Cunninghamella elegans*. *Arch. Environ. Contam. Toxicol.* **32**: 117–225.

Pothuluri, J.V., J.P. Freeman, T.M. Heinze, R.D. Beger, and C.E. Cerniglia (2000) Biotransformation of vinclozolin by the fungus *Cunninghamella elegans*. *J. Agric. Food Chem.* **48**: 6138–6148.

Rankov, V., and B. Valev (1977) Detoxification of the herbicide propachlor by certain microscopic fungi. *Acta Phytopathol. Acad. Sci. Hung.* **12**: 67–72.

Rao, A.V., and N. Sethunathan (1974) Degradation of parathion by *Penicillium waksmanii* Zaleski isolated from flooded acid sulphate soil. *Arch. Mikrobiol.* **97**: 203–208.

Rawson, D., and W. Gaisford (1990) Biosensors monitor the environment. *Control. Instrum.* **22**: 63–64.

Rekha, K., M.S. Thakur, and N.G. Karanth (2000) Biosensors for the detection of organo-phosphorus pesticides. *Crit. Rev. Biotechnol.* **20**: 213–235.

Rickard, R.W., and N.D. Camper (1978) Degradation of fluometuron by *Rhizoctonia solani*. *Pestic. Biochem. Physiol.* **9**: 183–189.

Rigas, F., V. Drista, R. Marchant, et al. (2005) Biodegradation of lindane by *Pleurotus ostreatus* via central composite design. *Environ. Int.* **31**: 191–196.

Ryan, T.P., and J.A. Bumpus (1989) Biodegradation of 2,4,5-trichlorophenoxyacetic acid in liquid culture and in soil by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Microbiol. Biotechnol.* **31**: 302–307.

Sahasrabudhe, S.R., and V.V. Modi (1987) Microbial degradation of chlorinated aromatic compounds. *Microbiol. Sci.* **4**: 300–303.

Salama, A.K.M. (1998) Metabolism of carbofuran by *Aspergillus niger* and *Fusarium graminearum*. *J. Environ. Sci. Health B* **33**: 252–266.

Savin, M.C., and J.A. Amador (1998) Biodegradation of norflurazon in a bog soil. *Soil Biol. Biochem.* **30**: 275–284.

Schilling, R., G. Engelhardt, and P.R. Wallnofer (1985) Degradation of the herbicide metribuzin (sencor) by pure cultures of *Cunninghamella echinulata* Thaxter ATCC 38447. *Chemosphere* **14**: 267–270.

Siegle-Murandi, F., R. Steiman, and J.-L. Benoit-Guyod (1991) Biodegradation potential of some micromycetes for pentachlorophenol. *Ecotox. Environ. Saf.* **21**: 290–300.

Siegle-Murandi, F., R. Steiman, J.-L. Benoit-Guyod, and P. Guiraud (1992) Biodegradation of pentachlorophenol by micromycetes. I. Zygomycetes. *Environ. Toxicol. Water Qual.* **7**: 125–139.

Singh, U.D., N. Sethunathan, and K. Raghu (1991) Fungal degradation of pesticides. In: *Handbook of Applied Mycology: Soil and Plants*, D.K. Arora, B. Rai, K.G. Mukerji, and G.R. Knudsen, eds. Marcel Dekker, New York, Vol. 1, pp. 541–588.

Spanis, W., and D.E. Munnecke (1962) Bacterial inactivation of organic fungicides. *Phytopathology* **52**: 365.

Spanis, W., D.E. Munnecke, and R.A. Solberg (1962) Biological breakdown of two organic mercurial fungicides. *Phytopathology* **52**: 455–462.

Sun, H.L., T.J. Sheets, and F.T. Corbin (1990) Transformation of alachlor by microbial communities. *Weed Sci.* **38**: 416–420.

Tekere, M., I. Ncube, J.S. Read, and R. Zvauya (2002) Biodegradation of the organochlorine pesticide, lindane by a sub-tropical white rot fungus in batch and packed bed bioreactor systems. *Environ. Technol.* **23**: 199–206.

Tessier, J. (1982) The path to deltamethrin. In: *Deltamethrin Monograph*. Roussel Uclaf, Paris, pp. 25–36.

Tillmanns, G.M., P.R. Wallnofer, G. Engelhardt, K. Olie, and O. Hutzinger (1978) Oxidative dealkylation of five phenylurea herbicides by the fungus *Cunninghamella echinulata*. *Chemosphere* **7**: 59–64.

Tomlin, C.D.S. (2000) *The Pesticide Manual*. British Crop Protection Council, Surrey, UK.

Torstensson, N.T., J. Stark, and B. Goransson (1975) The effects of repeated applications of 2,4-D and MCPA on their breakdown in soil. *Weed Sci.* **15**: 159–164.

Uesugi, Y., and C. Tomizawa (1971a) Metabolism of *O*-ethyl-*S*,*S*-diphenylphosphorodithioate (Hinosan) by mycelial cells of *Pyricularia oryzae*. *Agric. Biol. Chem.* **35**: 941–949.

Uesugi, Y., and C. Tomizawa (1971b) Metabolism of *S*-benzyl-*O*-ethylphenylphosphorothioate (Inezin) by mycelial cells of *Pyricularia oryzae*. *Agric. Biol. Chem.* **36**: 313–317.

USEPA (1991) Rules and regulations. *Fed. Reg.* **56**: 3552.

Vroumsia, T., R. Steiman, F. Seigle-Murandi, and J.-L. Benoit-Guyod (1996) Biodegradation of three substituted phenylurea herbicides (chlortoluron, diuron, and isoproturon) by soil fungi: a comparative study. *Chemosphere* **33**: 2045–2056.

Vroumsia, T., R. Steiman, F. Seigle-Murandi, and J.-L. Benoit-Guyod (1999) Effects of culture parameters on the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-dichlorophenol (2,4-DCP) by selected fungi. *Chemosphere* **39**: 1397–1405.

Vroumsia, T., R. Steiman, F. Seigle-Murandi, and J.-L. Benoit-Guyod (2005) Fungal bioconversion of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-dichlorophenol (2,4-DCP). *Chemosphere* **60**: 1471–1480.

Wang, J., S.A. Kane, J. Liu, M.R. Symth, and K. Rogers (1996) Mushroom tissue based biosensor for inhibitor monitoring. *Food Technol. Biotechnol.* **34**: 51–55.

Weinberger, M., and J.M. Bollag (1972) Degradation of chlorbromuron and related compounds by the fungus *Rhizoctonia solani. Appl. Microbiol.* **24**: 750–754.

Wolf, D.C., and J.P. Martin (1976) Decomposition of fungal mycelia and humic type polymers containing carbon-14 from ring and side chain labeled 2,4-D and chlorpropham. *Soil Sci. Soc. Am. J.* **40**: 700–704.

Wortberg, M., C. Middendorf, A. Katerkamp, T. Rump, J. Krause, and K. Cammann (1995) Flow-injection immunosensor for triazine herbicides using Eu(III) chelate label fluorescence detection. *Anal. Chim. Acta* **289**: 177–186.

Yadav, J.S., and C.A. Reddy (1993) Mineralization of 2,4-dichlorophenoxyacetic acid (2,4-D) and mixtures of 2,4-D and 2,4,5-trichlorophenoxyacetic acid by *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **59**: 2904–2908.

Yen, P.Y., W.C. Koskinen, and E.E. Schweizer (1994) Dissipation of alachlor in four soils as influenced by degradation and sorption processes. *Weed Sci.* **42**: 233–240.

Yokoyama, K., K.I. Ikebukuro, E. Tamiya, I. Karube, N. Ichiki, and Y. Arikawa (1995) Highly sensitive quartz crystal immunosensor for multisample detection of herbicides. *Anal. Chim. Acta* **304**: 139–145.

Zayed, S.M.A.D., I.Y. Mostafa, M.M. Farghaly, H.S. Attaby, Y.M. Adam, and F.M. Mahdy (1983) Microbial degradation of trifluralin by *Aspergillus carneus*, *Fusarium oxysporum* and *Trichoderma viride. J. Environ. Sci. Health B* **18**: 253–260.

Zherdev, A.V., N.A. Bizova, A.I. Yaropolov, N.V. Lyubimova, O.V. Morozova, and B.B. Dzantiev (1999) Laccase from *Coriolus hirsutus* as alternate label for enzyme immunoassay. *Appl. Biochem. Biotechnol.* **76**: 203–215.

7

FUNGAL METABOLISM OF PHENOLS, CHLOROPHENOLS, AND PENTACHLOROPHENOL

7.1 INTRODUCTION

Phenols are widely distributed in nature, especially in plants, where these occur in the form of alkaloids, coumarins, flavonoids, terpenes, tannins, and lignins. Phenols are also found in marine systems, produced by marine plants and animals, and are degraded by indigenous microbial populations. In 1998, 4.77 billion pounds of phenol was produced, with an expected increase of 3% per year, and ranked among the 40 most produced chemicals in the United States (ChemExpo, 1999). About 70% of industrially produced phenol is employed in the production of resins and is also used in the manufacture of plastics, biocides, disinfectants, textiles, medicines, explosives, inks, perfumes, photographic materials, and several other products.

Phenols and halogenated phenols arise as wastes or by-products of numerous industries, and their presence in waste streams is of environmental concern. Release of phenols in the environment is known in almost all industries that manufacture or use phenols. Phenolics of low molecular mass include the chlorophenols, guaiacol, chloroguaiacols, chlorocatechols, and chlorinated vanillic and syringic acids. Pentachlorophenol (PCP) is a general biocide whose worldwide use in wood and timber preservation has contributed severe contamination in wood-treatment plants. PCP is toxic to all forms of life because it is an inhibitor of oxidative phosphorylation. The well-documented toxicity and persistence of PCP in various media necessitate cleanup at

contaminated sites. Chlorophenols have also been used for wood preservation and a wide range of domestic, agricultural, and industrial purposes for more than 50 years and are presently widespread in the environment (Jensen, 1996). The occurrence of chlorophenols in aquatic and terrestrial food chains has also been established.

In addition to industrial production, chlorophenols are produced from naturally occurring phenols as a result of the bleaching of wood pulp. Phenol is regarded as a priority contaminant by the U.S. Environmental Protection Agency (Keith and Telliard, 1979). Creosote spills have contributed to its detection in river water (Ventura et al., 1998) and in effluents from wastewater treatment plants (Paxeus, 1996). The U.S. Environmental Protection Agency has set a limit of 600 µg/l as a 24-hour average to protect freshwater aquatic life, not to exceed 3.4 mg/l (36 µM), and a drinking water limit of 1 µg/l (0.01 µM) (USEPA, 1979). The Preliminary Remediation Goals (PRGs) for the Superfund and Resource Conservation and Recovery Act (RCRA) programs for phenol, 2-chlorophenol, and pentachlorophenol are set to the limits of 37 000 mg/kg, 63 mg/kg, and 3 mg/kg, respectively, for residential soils and 22 000 µg/l, 30 µg/l, and 0.56 µg/l, respectively, for groundwater (USEPA, 2000).

7.2   ALTERNATIVE TREATMENT TECHNOLOGIES

In recent years, attempts have been focused on developing highly effective technologies related to detoxification of phenols. Numerous conventional purification methods, such as solvent extraction, adsorption on activated carbon, and chemical oxidation, have been employed for the dephenolization of industrial wastewaters. Although these methods are effective, most suffer from serious drawbacks, due to high cost, incompleteness of purification, formation of hazardous by-products, and applicability to a limited phenol concentration range.

7.2.1   Physicochemical Methods

During the past decade, several types of oxidative methods have been tried for the removal of phenols from solutions or wastewaters. Phenol degradation by a photo-Fenton reaction has been demonstrated in highly concentrated wastewaters and the intermediate species can be identified by Fourier transform infrared (FTIR) spectroscopy with an attenuated total reflection (ATR) device (Arana et al., 2001). A global mechanism for photo-Fenton degradation of phenol has also been proposed. Phenol has been oxidized over CuO/Al_2O_3 in supercritical water (Yu and Savage, 2000) and $Pt_xAg_{1-x}MnO_2/CeO_2$ catalysts (Hamoudi et al., 2000). A heterogeneous copper catalyst supported on mesoporous MCM-41 with a pore area of over $1400 m^2/g$ showed high catalytic activity for the wet oxidation of phenol solution at a concentration

of 1300 ppm at 150 and 200°C (Wu et al., 2001b). An advanced oxidation process (AOP) of ultraviolet light/hydrogen peroxide has been investigated for the degradation of phenolic compounds in a completely mixed batch photolytic reactor (Alnaizy and Akgerman, 2000). Three isomers of chlorophenol were completely dechlorinated within 5 hours by palladium/iron powder in water through catalytic reduction (Liu et al., 2001). Bisphenol A can be oxidized electrochemically by voltammetric techniques (Kuramitz et al., 2001).

Phenol can be photo-degraded in aqueous solution by photocatalysis and direct photolysis (Chun et al., 2000). The two processes show different reaction pathways of phenol degradation. Photodegradation of phenol and 4-chlorophenol by $BaO–Li_2O–TiO_2$ catalysts (Leyva et al., 1998), photooxidation of 2,6-dimethylphenol and monophenylphenols in the presence of uranyl ion in water (Sarakha et al., 1997), and the role of metals in homogeneous photooxidation of phenols (Sykora et al., 1997) have been demonstrated. Photo-sonochemical degradation (Wu et al., 2001a) and photo-nitration of phenol (Vione et al., 2001a,b) in aqueous solution have also been evaluated. Jung et al. (1993) obtained a 30% increase in the degradation rate by applying a magnetic south pole to a bioreactor with immobilized microbial beads. Phenol has also been immobilized in cement-based solidified/stabilized hazardous wastes using regenerating activated carbon (Hebatpuria et al., 1999).

7.2.2 Biological Methods

7.2.2.1 Metabolism by Bacteria. Some phenol-degrading microorganisms have been listed and kinetic models described by Vipulanandan et al. (1994). *Pseudomonas* sp. D8 has the ability to degrade monochlorophenols, nitrophenols, and phenol (Chang et al., 1997). Resting cells of 2,4,5-trichlorophenol (2,4,5-TCP)-grown *Pseudomonas cepacia* AC1100 converted dichlorophenols, 2,4,6-trichlorophenol (2,4,6-TCP), and pentachlorophenol (PCP) to the corresponding chlorohydroquinones (Tomasi et al., 1995). *Alcaligenes eutrophus* assimilated phenol as a sole source of carbon and 4-chlorophenol as a cometabolite (Hill et al., 1996). *Pseudomonas putida* cometabolized 4-chlorophenol in the presence of phenol at various concentration ratios in an external-loop fluidized-bed airlift bioreactor (EFBAB) (Loh and Ranganath, 2005). *A. eutrophus* JMP134 degraded 2,4,6-TCP completely and 40% of 2,4,5-TCP (Clement et al., 1995). Bench-scale experiments have explored the feasibility of passive evaporation, soil washing, and biodegradation for treatment of phenol and cresol in the soil (Evangelista et al., 1990). Biotreatment experiments have found *A. eutrophus* JMP134 in soil. A full-scale soil-leaching process removed more than 99.9% phenol and 99.7% cresols.

Phenol-induced cells of *Acinetobacter* species have been shown to degrade 4- and 3-chlorophenols (4- and 3-CPs) (Kim and Hao, 1999). *Streptomyces*

rochei 303 assimilated a wider range of chlorinated phenols, such as dichloro-, tri-, tetra-, and pentachlorophenol, as the sole sources of carbon and energy (Golovleva et al., 1991–1992). *S. rochei* 303 showed the activity of hydroxy-quinol 1,2-dioxygenase in cell-free extracts. Degradation of 2,4,5-TCP and 2,3,5,6-tetrachlorophenol has been successful when efficient pulse electric discharge has been followed by bioremediation with a bacterial consortium from acclimated return activated sludge (Tartakovsky et al., 2001). Immobilized cells of *Pseudomonas putida* were shown able to degrade phenolic industrial wastewater in a fluidized-bed reactor (Gonzalez et al., 2001). Four species of bacteria exhibited an *ortho*-cleavage pathway to metabolize chlorophenols by immobilized cultures in a series of batch reactors (Lu et al., 1996). Three species effectively removed phenol at a concentration of 200 mg/l. *Pseudomonas* removed phenol in a four-stage cross-flow rotating biological contactor (RBC) reactor (Banerjee, 1997). Chlorophenol was mineralized 99% at loading rates of 1000 mg/l in high-rate fluidized-bed biofilm systems within less than an hour (Puhakka et al., 1995). Droste et al. (1998) showed the read removal of *ortho*-chlorine atoms and toxicity of *para*-chlorine atoms in chlorinated phenols using upflow-anaerobic sludge blanket (UASB) reactors.

7.2.2.2 *Metabolism by Actinomycetes.*

Strains of thermophilic/thermotolerant *Saccharomonospora viridis* were shown to have the ability to remove PCP from liquid cultures within 8 days (Webb et al., 2001). The PCP removal depends on growth and requires de novo protein synthesis. The pathway proposed indicates oxidation of PCP to tetrachlorohydroquinone (TCHQ), which undergoes further oxidation to tetrachloro-1,4-benzoquinone (TCBQ), and this forms polar conjugates to excrete into the growth medium. However, *S. viride* is unable to mineralize PCP in experiments conducted with [^{14}C]PCP. *Arthrobacter aurescens* TW17 degrades *para*-nitrophenol to hydroquinone and nitrite and *Nocardia* sp. strain TW2 to hydroquinone or 4-nitrocatechol (Hanne et al., 1993).

7.2.2.3 *Metabolism by Algae and Higher Plants.*

An isolated unicellular microalga, VT1, reduced 70.3% PCP from media in the presence of light (Tikoo et al., 1996). Mineralization appeared to occur due to evolution of $^{14}CO_2$ from [^{14}C]PCP by VT1. *Ochromonas danica* degrades phenol and its methylated homologs and shows catabolic versatility to degrade mixtures of phenols (Semple and Cain, 1996, 1997). The first *meta*-cleavage pathway for aromatic ring degradation in a eukarotic alga has been identified. Nearly 22% of pentachlorophenol mineralized after 155 days when crested wheatgrass (*Agropyron desertorum*) was vegetated on contaminated soil (Ferro et al., 1994). Glucose and glucuronic acids were produced by wheat (*Triticum aestivum*) cells after 2 days upon exposure to 1 mg/l of 4-*n*-nonylphenol (Bokern and Harms, 1997).

7.3 FUNGAL BIOSENSORS FOR DETERMINATION OF VARIOUS TYPES OF PHENOLS

Living organisms are used as biological indicators to examine the toxicity of chemical substances for the last four decades. *Armillaria mellea* and *Mycena citricolor* have been employed to develop a novel, bioluminescence-based toxicity bioassay of 3,5-dichlorophenol (3,5-DCP) and PCP within 60 minutes (Weitz et al., 2002). Light reduction was observed in response to 3,5-DCP and PCP for both fungi. The EC_{50} values for both fungi were similar to the EC_{50} values for 3,5-DCP and PCP of bioluminescence-based bacterial biosensors. Different biosensor configurations, including enzyme electrodes, catalytic cycles, and mechanisms for the determination of phenolic compounds, have been described (Marko-Varga et al., 1995). Nistor and Emneus (1999) described the biosensors for monitoring the polar organic pollutants, including the phenolic compounds, together with 200 references.

7.3.1 Enzyme-Based Systems

Several enzymes possess the ability to convert specific or less specific compounds in the presence of oxygen or can generate other compounds. The extent of consumption or generation depends on the concentration of converted compound. Certain compounds can also act as inhibitors of activity of enzymes, and the extent of inhibition can be determined. These tools can be classified into three types: type 1 for highly selective detection of specific target compounds, type 2 for screening purposes, and type 3 as a biomarker of toxicity and for global toxicity monitoring (Wollenberger et al., 1994). Fungal biosensors for the determination of various types of phenols are noted in Table 7.1.

Several strains of *Trichosporon* yeasts have been utilized in biosensors for environmental purposes (Neujahr, 1984, 1990). Three different biosensors have been constructed based on the reduction of oxygen during phenol degradation by *Trichosporon cutaneum* using platinum–silver Clark-type oxygen electrodes. Various tissues and cells rich in phenol oxidases, such as mushroom (Skladal, 1991; Canofeni et al., 1994) or laccase (Ghindilis et al., 1992), have been used in combination with Clark-type oxygen electrode. Purified (Kjellen and Neujahr, 1980) and immobilized (Kjellen and Neujahr, 1979) phenol hydroxylase electrodes have also been employed for the detection of phenol.

Co-immobilizing enzymes with different specificities for phenolic compounds is another approach to improving the sensitivity of biosensors. Tyrosinase–peroxidase (Cosnier and Popescu, 1996) and tyrosinase–laccase (Yaropolov et al., 1995) biosensors have been discussed. Ultrasensitive biosensors have been developed by combining tyrosinase (Bier et al., 1996a) or laccase (Bier et al., 1996b) with either quinoprotein glucose dehydrogenase

TABLE 7.1 Fungal Biosensors for Determination of Phenols

Fungus/Yeast	Biocomponent	Electrode Material(s)	Constituent(s)	Detection Limit	Reference
Saccharomyces cerevisiae	Cells	Ferricyanide amperometric sensor	Phenol	0.1–1.5 mM/l	Gaisford et al., 1991
S. cerevisiae	Immobilized cells	Amperometric gas diffusion oxygen electrode	Phenol	0.1 mM/l	Campanella et al., 1995
Mushroom	Tyrosinase immobilized in polyvinyl alcohol membrane	Clark-type oxygen electrode	Catechol, phenol, cresol, chlorophenol, etc.	$0.6 \, mM/dm^3$ $0.9 \, mM/dm^3$	Makower et al., 1996
Mushroom	Tyrosinase	Thick-film electrode carbon ink	Phenol	100 nM	Wang and Chen, 1995
Mushroom	Tyrosinase	Graphite epoxy carbon paste	Phenols	40–100 nM	Onnerfjord et al., 1995
Trametes versicolor	Laccase	Immobilized on carbon fiber electrode	Catechol	1–90 μM/l	Freire et al., 2001
Mushroom and *Coriolus hirsutus*	Tyrosinase and laccase	Immobilized onto a solid graphite electrode	Phenol, catechol, cresol, hydroquinone, etc.	1 mM	Yaropolov et al., 1995
Trichosporon cutaneum	Purified phenol hydroxylase	Platinum–silver Clark-type electrode	Phenol	0.5 μM	Kjellen and Neujahr, 1980

or oligosaccharide dehydrogenase on an oxygen electrode. Fluorimetric determination of phenols has been developed by an immobilized tyrosinase amplification flow injection analysis (FIA) system (Fuhrmann and Spohn, 1998). The detection limit is $0.02\,\mu M$ for both phenol and catechol. Peroxidase biosensors can also be used for the determination of phenols (Ruzgas et al., 1995; Munteanu et al., 1998).

7.3.2 Biological Affinity Assays (Immunoassays)

Biological affinity assays were developed for medical purposes, but many of them are now being evaluated for possible use in environmental applications. These include batch immunoassays, immunobiosensors, and flow immunoassays. Most of them are enzyme-linked immunosorbent assays with either the antigen (Ag-indirect ELISA) or the antibody (Ab-direct ELISA) immobilized to either plates or particles. The presence of 1-naphthol can be detected in groundwater by use of ELISA type Ia (Marco et al., 1995). Direct Ab-ELISA type IIa has been developed for the determination of PCP (Gerlach and Van Emon, 1997).

7.4 METHODS OF ANALYSIS OF VARIOUS TYPES OF PHENOLS

The phenolic compounds can be separated and confirmed by HPLC and TLC and by HPLC, gel permeation chromatography, and TLC-MS. The metabolites of 4-(1-nonyl)phenol are analyzed by TLC on silica gel plates with a fluorescent indicator (Vallini et al., 2001), and those of phenolic compounds by TLC, HPLC, and MS (Edlin et al., 1995). Phenolic compounds have been separated by HPLC with absorbance and fluorimetric detectors in series (Rodriguez-Delgado et al., 2001). Eleven Environmental Protection Agency (EPA) priority phenols (POHs) have been separated and determined by reverse-phase capillary HPLC with indirect fluorophotometric detection (Chung and Lee, 2001). The bioconversion of phenol and *para*-cresol has been measured by reverse-phase HPLC using software and a UV detector (Luke and Burton, 2001). Progress in the development of microchip capillary electrophoresis (CE) with electrochemical (EC) detection, and the advantages, have been described (Lacher et al., 2001). *Candida nitrativorans* degraded radiolabeled phenol in internal and external collection systems in a study by Bastos et al. (1997). Use of an external collection system for radiorespirometric measurement of an aerobic process using a substrate has been recommended.

7.5 FUNGAL BIOREACTORS FOR REMOVAL OF VARIOUS TYPES OF PHENOLS

Recently, fungal bioreactors have been developed for the removal of phenols, chlorophenols, and PCP. These bioreactors are still in the developmental

phase, and thus scaling in the light of different optimum operating conditions is necessary. Logan et al. (1994) suggested some factors for the conceptual design of fungal PCP bioreactors: (1) long detention times of 10 to 40 days from inoculation to complete PCP removal, (2) study of chemical adsorption on a case-by-case basis, and (3) the insignificance of a system design with nitrogen limitations in the bioreactor feed and the significance of a system design to produce shallow fluid interfaces.

Commercial applications of these bioreactors in the metabolism of phenols, chlorophenols, and PCP are unknown at this time. However, based on the limited research, fungal bioreactors (Table 7.2) are classified into four types, described below.

7.5.1   Rotating Tube Bioreactors

A rotating tube bioreactor (RTB) has been developed to study degradation of PCP by fixed films of white-rot fungi (Alleman et al., 1995). Tubes were rotated to expose the mycelial biofilm on the mesh to a wetting–aeration cycle characteristic of fixed-film reactors such as trickling filters and rotating biological contactors. All species of fungi grew well in the RTBs and showed thick biofilms, which remained attached to the mesh during tube rotation. *Trametes versicolor* showed the highest dehalogenation (62%), followed by *Phanerochaete chrysosporium* and *Inonotus dryophilus*, after 8 days. The total organic halide (TOX) concentrations decreased in the order *I. dryophilus* (1.8 mg Cl/l), *P. chrysosporium* (0.5 mg Cl/l), and *T. versicolor* (0.3 mg Cl/l). About 33% of organically bound chlorine (OBX) was contained within the mycelia of *T. versicolor*, 55% of *P. chrysosporium*, and 52% of *I. dryophilus*.

7.5.2   Membrane Bioreactors

Membrane bioreactors designed for cell immobilization are now employed in the detoxification of industrial phenolic wastewater. A novel polysulfone capillary membrane bioreactor system with immobilized fungal biofim of *Neurospora crassa* (Figure 7.1) was used to degrade phenol or *para*-cresol (Luke and Burton, 2001). A capillary bioreactor was operated in a dead-end configuration and showed a high percentage removal of phenol and *para*-cresol. Laccase production was sustained at a level of 10 U/ml of permeate (1080 U/g wet biomass) over 30 to 40 days. This was the first report on the continuous production of laccase by *N. crassa* grown in immobilized systems. The biodegradative capacity of the bioreactor was maintained continuously to sustain this removal efficiency for 4 months.

7.5.3   Packed-Bed/Immobilized Bioreactors

The use of immobilized fungi has several advantages, such as prolonged retention of fungal cells or enzymes with minimal washout even at high dilution

TABLE 7.2 Fungal Bioreactors for Removal of Phenols, Chlorophenols, and Pentachlorophenol

Fungus	Bioreactor Type	Bioreactor Volume	Constituent Conc.	Removal Rate (%)	Enzyme Activity	Duration (days)	Reference
Phanerochaete chrysosporium *Trametes versicolor* *Inonotus dryophilus*	Rotating tube	200×25 mm culture tube	PCP, 10 mg/l	38 62 21		8	Alleman et al., 1995
Neurospora crassa	Capillary membrane, immobilized biofilm	Membranes (o.d. 2 mm), each 12 cm in length	Phenol *p*-Cresol, 1.0 mM	100 92	Laccase, 10 U/ml	6	Luke and Burton, 2001
Trichosporon cutaneum	Immobilized to polyamide granules (chemically modified)	Cultivation of immobilized cells in 500-ml flask	Phenol, 1 g/l	>99		3	Godjevargova et al., 1998
P. chrysosporium	Biocarrier packed bed	8.0 L	PCP, 30 mg/l	>80	LiP, 12 U/ml MnP, 4.9 U/ml	12	Shim and Kawamoto, 2002
P. chrysosporium	Polyurethane foam and polyethylene immobilization, RBC, immobilized on polyethylene disks	47 cm in length 18 cm in diameter	4-CP, 100 mg/l	100 100		4 2	Zouari et al., 2002
Three white-rot fungi	Trickling packed bed Pinewood reactor (1) Foam glass reactor (2)	3540 cm^3 2450 cm^3	Phenol 2,4,6-TCP Phenol 2,4,6-TCP	99 98 99 100		2–3	Ehlers and Rose, 2005
Penicillium camemberti	Upflow column	6.7 cm (inner dia.), 55.7 cm height	PCP (AOX 63.36 mg/l)	77	LiP, $16.7–19 \, l^{-1}$	4	Taseli and Gokcay, 2005

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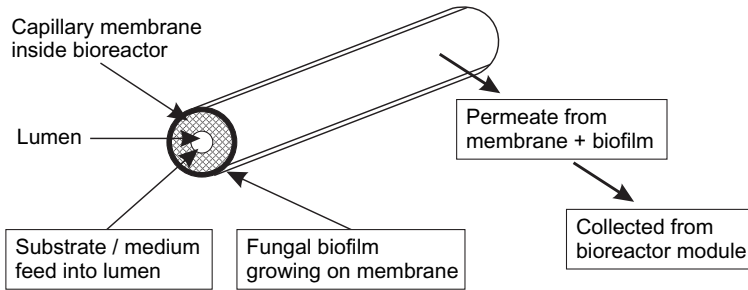
Figure 7.1 Capillary bioreactor system with immobilized *Neurospora crassa* biofilm. [Reprinted from Luke and Burton (2001), copyright © with permission from Elsevier.]

rates. Most of the reactors discussed earlier suffer limitations in maintaining a consistent high level of enzyme activity, steady-state continuous enzyme production and fungal activities for prolonged periods, and controlled fungal growth. A major problem lies in the lack of a suitable bioreactor system and immobilization technology. However, several studies of reactor systems involving white-rot fungi have been described (Shim and Kawamoto, 2002). Phenol can be degraded long term by *Trichosporon cutaneum* R57 covalently immobilized to polyamide granules with a high surface area (Godjevargova et al., 1998). Covalently bound cells degrade phenol at concentrations of 0.5 and 1.0 g/l in less time (24 hours) than do free cells (27 hours). Phenol is fully degraded in 27 hours for the first five cycles and in 36 hours for the next four cycles (from sixth to ninth), and degradation decreases to 80% during the tenth cycle. *T. cutaneum* cells bound to cellulose granules have the capacity to degrade furfurol (Ivanova and Yotova, 1993).

The packed-bed reactor system depicted in Figure 7.2 has been employed for the degradation of PCP by two wild-type strains of *Phanerochaete chrysosporium* (Shim and Kawamoto, 2002). Of five biocarriers, the polyurethane foam produced the highest biomass. Biostage and Biolace show similar high levels of growth and adhesion. The Biostage carrier produced the highest LiP activity of 22.6 U/ml on day 11 with an average LiP activity of 12 U/ml and an average MnP activity of 4.9 U/ml through 7 to 21 days. Steady-state continuous degradation of PCP over an extended period reached a degradation ratio exceeding 80%. The major metabolites were identified as 2,4,6-TCP and 2,3,4,6-TCP in a Biostage packed-bed reactor after 12 days. Polyurethane foam and polyethylene were used as carriers for the immobilization of *P. chrysosporium* in static and agitated cultures for 4-chlorophenol (4-CP) degradation (Zouari et al., 2002). These carriers allowed total 4-CP disappearance within 4 and 6 days for the first and second batches and 90% for the third batch within 12 days. Immobilization on polyethylene disks in RBC depletes total 2-CP within 2 days. Degradation of 4-CP occurred in the presence of a high-glycerol and high-glutamate medium which is known to
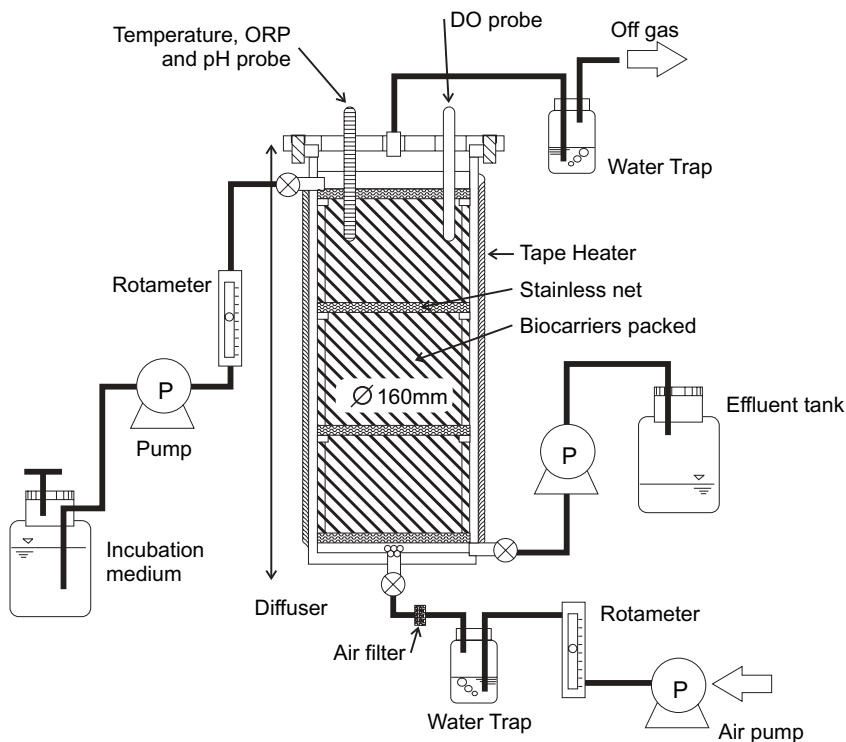
Figure 7.2 Packed-bed bioreactor system using strains of *Phanerochaete chryso-sporium*. [Reprinted from Shim and Kawamoto (2002), copyright © with permission from Elsevier.]

suppress the lignin-degrading system, suggesting that LiP and MnP are not involved directly in 4-CP degradation. Three immobilized white-rot fungi in pinewood chip and foam glass packed-bed trickling reactors operated in a sequencing batch eliminated 98 to 100% of influent phenol and 2,4,6-TCP (800 and 85 mg/l) in 24 to 30 hours (Ehlers and Rose, 2005). Higher LiP activity has been detected in a foam glass packed-bed reactor than in a pinewood chip reactor.

7.5.4 Upflow Column Bioreactors

A bench-scale upflow tubular column reactor removed 77% PCP from adsorbable organic halogen (AOX) by *Penicillum camemberti* (Taseli and Gokcay, 2005). In addition to the column, the reactor consisted of a feed tank, a feed pump, an inlet, an outlet, and four sampling outlets. The constant hydraulic loading rate of the feed solution is 2.12 l/l per day. The reactor is operated without aeration and a minimum amount of carbon supplement.

7.5.5   Miscellaneous Bioreactors

A wide variety of composting systems are well established, including circular tanks, in-ground trenches, open bins, rotating drums, windrows and open piles, and silos. Most composting systems utilize bulking agents, such as bark chips, straw, and chopped sugar beet, that augment the porosity and oxygen content in the medium and reduce the moisture levels. Despite limitations, composting has proven advantages. The fate of organic pollutants in soil-composting/compost systems has been discussed (Semple et al., 2001). All composting systems showed about 50 to 60% mineralization of [UL-^{14}C]PCP after 30 days (Laine and Jorgensen, 1997). Windrow systems were also employed for pilot-scale composting of 44 mg/kg chlorophenol-contaminated soils using different inoculants. Nearly 80% of chlorophenols was eliminated after 2 months. At this stage, highly contaminated soils of 683 to 1108 mg/kg were added to the composting windrows. Finally, more than 90% of chlorophenols were eliminated after 3 months.

7.6   FUNGAL METABOLISM OF PHENOLS, CHLOROPHENOLS, AND PENTACHLOROPHENOL

Research on fungal degradation of different groups of phenols is well established during the last three decades. However, it is important to find methods for the selection and isolation of phenol-degrading fungi. Bastos et al. (1996) stated a method for microbial selection based on adaptation to phenol and isolation of microorganisms to utilize phenol as a sole source of carbon. Labeled phenol (^{14}C) can also be used to determine the relative degradative capacities of microbial communities and isolates. Moreover, phenol-degrading microorganisms can be employed exogenously in chemically stressed habitats. Fungal degradation of phenols, chlorophenols, and pentachlorophenol is noted in Table 7.3.

7.6.1   Fungal Metabolism of Phenols

In general, phenols are metabolized to catechol by bacteria and fungi, and catechol leads to further degradation. Based on the growth of 88 Micromycetes belonging to different taxonomic groups, Rahouti et al. (1995) reported the best fungal degradation of compounds devoid of a carboxyl group and did not correlate with phenol oxidase activity. Several species and strains of Micromycetes provided the highest growth on protocatechuic and vanillic acids as the sole sources of carbon (Rahouti et al., 1999). Nearly 74% strains showed good or medium growth. Syringic and ferulic acids provided good growth, 65% and 62% of strains, respectively, followed by poor growth on catechol (52%) and guaiacol (45%) and low growth on phenol (37%). Phenolic compounds with —OH or —COOH substituents were easier to metabolize

TABLE 7.3 Fungal Metabolism of Phenols, Chlorophenols, and Pentachlorophenol

Fungus/Yeast	Phenolic(s) Conc.	Medium	Degradation/ Mineralization Rate (%)	Duration (days/hours)	Reference
Coprinus sp.	Phenol, guaiacol, ferulic acid, PCP, conc. 0.05%	GS liquid medium	94, 100, 100, 56	5 d	Guiraud et al., 1999
Coprinus cinereus	Phenol, guaiacol, ferulic acid, PCP, conc. 0.05%		96, 98, 80, 35		
Coprinus micaceus	Phenol, guaiacol, ferulic acid, PCP, conc. 0.05%		98, 87, 62, 35		
Phanerochaete chrysosporium	Phenol, 500 mg/l	GS liquid medium	90	5 d	Krivobok et al., 1994
Graphium sp. FIB4	Phenol, 10 mM	Liquid mineral medium	75	7 d	Santos and Linardi, 2004
Candida tropicalis	Phenol 1000 mg/l 1800 mg/l	Mineral medium	100 100	32 h 59 h	Yan et al., 2005
Pleurotus ostreatus	Bisphenol A, 0.4 mM	Liquid medium	80	12 d	Hirano et al., 2000
P. chrysosporium	*m*-Chlorophenol, 50 and 100 ppm *o*-Chlorophenol, 50 and 100 ppm	N-limited cultures	100, 100 100, 100	15 d	Perez et al., 1997
P. chrysosporium	2,4,5-[^{14}C]TCP (0.01 μCi/μM)	N-limited culture N-sufficient culture	61 of $^{14}CO_2$ 8 of $^{14}CO_2$	36 d	Joshi and Gold, 1993
Pleurotus cornucopiae	2,4,5-TCP or 2,4,6-TCP, 10 mg/l	N-sufficient medium	<25 <25	5 d	Seeholzer-Nguyen and Hock, 1991

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TABLE 7.3 Continued

Fungus/Yeast	Phenolic(s) Conc.	Medium	Degradation/ Mineralization Rate (%)	Duration (days/hours)	Reference
P. chrysosporium	2,6-DCP	N-limited medium	>99	3 d	Wu et al., 1996
		N-sufficient medium	>99		
P. ostreatus	2,4-DCP, 100μM	Liquid medium	100	10h	Rodriguez et al., 2004
P. pulmonarius			100	10h	
P. eryngii			100	24h	
P. sajor-caju			100	24h	
Chrysosporium pannorum	2,4-DCP, 100mg/l	GS synthetic liquid medium	61	7 d	Vroumsia et al., 2005
Mucor genevensis	2,4-DCP, 100mg/l		65		
Rhodotorula glutinis	Phenol, 5 mM	Mineral salts medium	100	18h	Katayama-Hirayama et al., 1994
	4-Chorophenol, 0.5mM		100	24h	
	3-Chlorophenol, 0.5mM		100	24h	
	2-Chlorophenol, 0.5mM		22	24h	
Oidiodendron echinulatum	PCP, 100mg/l	GS liquid medium	64	5 d	Seigle-Murandi et al., 1995
Calcarisporium arbuscula			88		
Strains of *Absidia*			69–88		Seigle-Murandi et al., 1992
Strains of *Cunninghamella*			62–90		

than those with —OCH3 substituents. Two species (*Paecilomyces variotii* and *Pestalotia palmarum*) among 1044 Micromycetes exhibited weak and strong phenol oxidase activity, respectively. Three species of *Coprinus* metabolized most phenolic compounds efficiently, except syringic acid (Guiraud et al., 1999). *C. cinereus* produced peroxidases, and *C. micaceus* mostly laccases. The phenol oxidase activity was very strong in *C. micaceus*, followed by *C. cinereus* and no response in *Coprinus* sp. PCP was nonassimilated by *Coprinus* sp. because of strong adsorption on mycelial biomass. Laccases and peroxidases from *Coprinus* species were not necessary for the degradation of these compounds. However, the purified peroxidase from *Coprinus* was involved in the metabolism of ferulic acid (Abelskow et al., 1997) and guaiacol (Morita et al., 1988).

Only 61 strains of 809 grow well in solid media, and these belong to the genera *Penicillium*, *Fusarium*, *Mucor*, *Absidia*, and *Epicoccum* (Krivobok et al., 1994). *Phanerochaete chrysosporium* depleted 90% of phenol at a high concentration at 37°C after 5 days. The first step was 2-hydroxylation of phenol to catechol after 1 day, followed by *ortho*-fission of the aromatic ring to produce muconic acid after 3 days that further oxidized to β-keto-adipate after 5 days. These metabolites were also quantified. FIB4, LEA5, and AE2 strains of *Graphium* sp. and FE11 of *Fusarium* sp. showed high rates of phenol degradation (Santos and Linardi, 2004). All strains secreted catechol 1,2-dioxygenase and phenol hydroxylase, suggesting the oxidation of catechol by *ortho*-type ring fission. Cain (1980) discussed the *ortho*-fission of aromatics in fungi. Degradation of *para*-cresol up to 150 mg/l was found in the presence of glucose and nitrogen-limited conditions using *Phanerochaete chrysosporium* BKM-F-1767 (Kennes and Lema, 1994). Two uptake systems were identified for phenol in glycerol-grown *Trichosporon cutaneum* (Mortberg et al., 1988). The first was an inducible, high-affinity system that is sensitive to protonophores. It was induced in coordination with phenol hydroxylase and can operate independent of phenol metabolism. The second was a constitutive low-affinity system with different specificities and was nonsensitive to protonophores. Phenol was also used as a sole carbon source and oxidized to catechol by *T. cutaneum* (Spanning and Neujahr, 1990). The soil-borne yeast *Candida maltosa* utilized phenol and catechol as the sole sources of carbon and energy at concentrations up to 1.7 g/l and 1.5 g/l, respectively (Fialova et al., 2004). An Aqualytic Sensomat System using BOD measurements was used to rapidly monitor the biodegradation. Phenol hydroxylase activity reached a maximum value during the complete degradation of phenol. The yeast also metabolized *para*-cresol. Phenol was also assimilated by hydrocarbon-utilizing yeasts (Hofmann and Schauer, 1988). Middelhoven et al. (1992a) reported the assimilation of phenol, catechol, resorcinol, hydroquinone, and some benzoic and cinnamic acid derivatives by several yeast strains, such as *Schizoblastosporion starkeyi-henricii* G28, *Trichosporon moniliforme* G41, *T. dulcitum* G37, *Cryptococus humicolus* G29, *C. laurentii* G30, *Leucosporidium scottii* G43, and *Rhodotorula*

aurantiaca G36. The growth of ascomycetous and basidiomycetous yeasts and yeastlike fungi on benzene compounds and the catabolic pathways has also been reviewed (Middelhoven, 1993).

7.6.2 Fungal Metabolism of Chlorophenols

Complete degradation of *meta-* and *para*-chlorophenol by *Phanerochaete chrysosporium* was shown to occur after 15 days (Perez et al., 1997). The degradation ability of *P. chrysosporium* depends on the position of substituent chlorine, and the *ortho*-position is the preferred attack position. The acclimated mycelium enhances the degradation rates of chlorophenols as well as more or less increasingly similar for three isomers, and the preference for *ortho*-position attack is reduced. *P. chrysosporium* degraded 2,6-dichlorophenol (2,6-DCP) at the same rate in N-limited and N-sufficient media (Wu et al., 1996). 2,6-DCP disappeared within 3 days in zero-order reaction rates. Biodegradation causes about 80% removal of 2,6-DCP, excluding bioadsorption and volatilization. No clear relationship exists between the ligninolytic enzymes and biodegradation of 2,6-DCP. Both LiP and MnP are detected in N-sufficient media and MnP only in N-limited medium. Of four species, *Pleurotus sajor-caju* showed the highest 2,4-DCP mineralization rate of 2% after 15 days (Rodriguez et al., 2004). Catechol and muconolactone were identified during complete assimilation of phenol by *Rhodotorula glutinis* (Katayama-Hirayama et al., 1994). The formation of muconolactone suggests *ortho*-fission of the ring as the preferable mechanism. 3-Chlorophenol (3-CP) and 4-chlorophenol (4-CP) are also well degraded with the stoichiometric release of chloride ions, followed by low degradation of 2-chlorophenol with a small release of chloride ions. Phenol or CP(s) act as inducers for the metabolism of 3-CP and 4-CP. GC/MS analysis revealed 4-chlorocatechol and maleylacetic acid as metabolites of 3-CP and 4-CP, and dechlorination may occur between the formation of 4-chlorocatechol and maleylacetic acid.

Fungi are also known to metabolize trichlorophenols (Seeholzer-Nguyen and Hock, 1991). GC analysis demonstrated a reduction of 25% of 2,4,5-TCP or 2,4,6-TCP within 4 to 5 days of inoculation by *Pleurotus cornucopiae*. In experiments with [^{14}C]-2,4,5-TCP, only 56% radioactivity was recovered after 6 days; what remained mineralized to CO_2. This suggests the degradation of trichlorophenols under conditions that are not nitrogen-limited. Such conditions favor the production of aryl-alcohol oxidase as well as the degradation of 2,4,5-TCP by other species of *Pleurotus*, such as *P. ostreatus florida* and *P. eryngii*.

7.6.3 Fungal Metabolism of Pentachlorophenol

Two new local strains of white-rot fungi have a good appetite for PCP in growth medium (Tayal et al., 1999). HPLC analysis indicated PCP removal

of 38.8% and 18.8% by strains Wd1 and I6, respectively, after 15 days. The biodegradation rate was higher in the initial phase and followed pseudo-first-order kinetics. Degradation of PCP was enhanced in the presence of Mn(II), H_2O_2, and glucose in the medium by strain Wd1. Strain Wd1 showed a maximum of 68% degradation in 16 days. This strongly suggests the involvement of peroxidase enzymatic system in the biodegradation of PCP.

A rapid disappearance of PCP from the medium by *Oidiodendron echinulatum* and *Calcarisporium arbuscula* after 5 days suggests strong adsorption on the mycelia (Seigle-Murandi et al., 1995). Logan et al. (1994) showed the extent of adsorption as a function of fungal species and PCP concentration. Degradation of PCP by Micromycetes (Ascomycetes, Basidiomycetes, yeasts, and Zygomycetes) has been examined (Seigle-Murandi et al., 1992, 1993; Steiman et al., 1994). Ascomycetes show heterogeneous results as the disappearance of PCP occurs both in phenol oxidase-producing and oxidase-nonproducing strains. Basidiomycetes deplete PCP moderately, irrespective of high producers of phenol oxidases. Poor degradation occurs in yeasts and phenol oxidases are not produced. *Absidia* and *Cunninghamella* are the most effective genera among 70 strains of Zygomycetes. No correlation exists between the production of phenol oxidases and PCP depletion among genera in all classes of Micromycetes. Mycelia of fungi such as *Armillaria*, *Ganoderma*, *Pleurotus*, *Polyporus*, *Coprinus*, and *Volvariella* were shown to have the ability to remove PCP in a batch cultivation system (Chiu et al., 1998). *Polyporus* mycelia exhibited the best PCP-absorbing capacity (31 g/kg), and *Armillaria* revealed the highest capacity (13 g/kg) to break down the compound.

7.7 FACTORS AFFECTING FUNGAL METABOLISM OF VARIOUS TYPES OF PHENOLS

The acceleration and effectiveness of phenol metabolism depend on the dynamics of the physicochemical factors of the medium or in the field. These factors include temperature, pH, aeration, type of agitation, type of inoculum, and carbon and nitrogen sources. The optimum requirement of these interdependent factors is important for the intensity of the detoxification process. The concentration and structure of the toxic substrates also affect the biodegradation activity. Various factors known to influence the effectiveness of phenol biodegradation are noted in Table 7.4.

7.7.1 Effect of Static Versus Agitated Culture Conditions

Both static and shaking cultures of *Neurospora crassa* are effective for the removal of *para*-cresol over a wide concentration range (Luke and Burton, 2001). Extracellular laccase activity was 10 to 12 U/ml in static and 1.5 U/ml in shaken cultures, respectively, over 8 to 15 days. Polyphenol oxidase was

TABLE 7.4 Factors Affecting Fungal Metabolism of Phenols, Chlorophenols, and Pentachlorophenol

Fungus	Phenolic(s) Conc.	Factors	Medium	Degradation/ Mineralization Rate (%)	Duration (days)	Reference
Neurospora crassa	*p*-Cresol, 0.5, 1.5, 2.5, and 5mM	Shaken cultures	Liquid medium	70, 79, 64, 91	6	Luke and Burton, 2001
		Static cultures		92, 92, 89, 67		
	Phenol, 0.5, 1.5, 2.5, and 5mM	Shaken cultures		100, 42, 67, 100		
		Static cultures		48, 46, 99, 99		
Penicillium camemberti	PCP 2-Chlorophenol	Shaken cultures	Basal salts medium	86 53	21	Taseli and Gokcay, 2005
Phanerochaete chrysosporium	PCP, 8mg/l	50-ml-deep-cultures	Liquid medium	>50	1	Logan et al., 1994
Trametes versicolor				>50		
Ganoderma (four species)				>50		
Inonotus rickii				96	10	
P. chrysosporium	PCP, 8mg/l	Culture age (60–70h)	50-ml-deep culture, N-deficient medium	80	20	
Inonotus dryophilus	PCP, 10mg/l	N-sufficient N-deficient	Shallow 10-ml cultures	100 100	10 10	

Species	Chemical	Conditions	Medium			Reference
T. versicolor		N-sufficient		100	6	
		N-deficient		100	10	
Ganoderma oregonense		N-sufficient		>98	10	
		N-deficient		>96	10	
Phoma glomerata	PCP, 100 mg/l	Light, 4800 lux 12 h/day	GS liquid medium	22	1	Seigle-Murandi et al., 1995
		Dark		54	2	
				14	1	
				31	12	
Deuteromycetes (12 strains)	PCP, 1 g/l	Glucose 5 g/l	GS liquid medium	12–18	6	
P. chrysosporium	*o*-Chlorophenol, 100 ppm	Glucose 10 and 2.5 g/l	N-limited	100, 100	15, 22	Perez et al., 1997
	m-Chlorophenol, 100 ppm	Glucose 10 and 3.5 g/l	static cultures	100, 100	15, 20	
	o-Chlorophenol, 100 ppm	Ammonium tartrate	N-limited	100	15	
	m-Chlorophenol, 100 ppm	0.2 g/l and glucose 0.1 g/l	static cultures	100	15	
	p-Chlorophenol, 100 ppm			100	15	

produced as an intracellular enzyme at 374 U/g wet mass. Under static conditions, 85 to 100% PCP was reduced with 10 to 50% recovery of $^{14}CO_2$ (Mileski et al., 1988). Magnetic stirring and orbital shaking influenced the depletion of PCP using 12 strains of Deuteromycetes (Seigle-Murandi et al., 1995). Half of the strains favored the depletion of PCP by an orbital type of agitation. Mycelia age and agitation speed affected PCP degradation in submerged cultures by *Rhizopus nigricans* (Cortes et al., 2001). A degradation efficiency of 0.028 mg PCP/mg biomass per hour was obtained under these conditions. The depth of fluid media also plays an important role in pollutant degradation by fungi (Logan et al., 1994). Of four species of fungi, *Inonotus rickii* exhibits nearly complete PCP removal after 6 days when grown in deep 50-ml suspensions in 250-ml flasks compared to PCP removal after 10 days when grown in shallow 10-ml suspensions. This indicates the importance of the ratio of surface area to volume of liquid media (oxygen transfer) in the extent of PCP removal by white-rot fungi.

7.7.2   Effect of Culture Age, Type of Inoculum, and Carbon and Nitrogen Sources

Glucose concentration (5 to 10 g/l) and type of inoculum (spores or mycelium plus spores) influence the depletion of PCP using 12 strains of Deuteromycetes (Seigle-Murandi et al., 1995). Glucose concentration of 5 g/l supported the best depletion of PCP. Spores of half of the strains did not develop a mycelial mat in 48 hours of cultivation to circumvent the toxic effect of PCP. These findings were also reported by Mileski et al. (1988). Better PCP depletion occurred in mycelia plus spores type of inoculums. Light (4800 lux, 12 hours per day) supported better depletion of PCP (22%) by *Phoma glomerata* after 24 hours than did dark.

The exposure time has been studied in relation to PCP, culture age, and C/N ratio in culture media by white-rot fungi (Logan et al., 1994). In most species, the rate of PCP removal decreases with time. The PCP (80%) degradation increased with culture age (60 to 70 hours) in N-deficient media, suggesting the potential removal of PCP by older cultures. The production time, concentration, and types of ligninolytic enzymes in *Phanerochaete chrysosporium* depend on the age of the culture (Leisola et al., 1987). So it is important to employ cultures of different growth stages and ages to maximize pollutant degradation and removal. Nitrogen-sufficient 20-day-old cultures dehalogenated slightly greater removal of PCP than did nitrogen-deficient cultures (Alleman et al., 1995). Recovery of chloride in nitrogen-sufficient and nitrogen-deficient cultures, respectively, were *P. chrysosporium*, 0.65 and 1.5 mg/l, *I. dryophilus*, 0.2 and 0.9 mg/l, and *T. versicolor*, 2.5 and 2.8 mg/l.

P. chrysosporium grown in either a 2% ammonium lignosulfonate (LS) (nitrogen-sufficient) medium or a 0.23% LS and 2% glucose (nitrogen-deficient) medium eliminated 72 to 75% of PCP after 3 days (Aiken and Logan, 1996). Dehalogenation of PCP took place despite inhibition of LiP activity by

LS. Only 1.85% of initial PCP recovered as PCP after 13 days. The remainder of PCP either mineralized or converted to intermediates in the form of organic halides. About 58% of original chlorine was recovered as organic, non-PCP halide, 73% of which was associated with cell mass. Of the remaining chlorine, 40% was released as chloride ion. Carbon and nitrogen concentrations influenced chlorophenol degradation by acclimated mycelium of *P. chrysosporium* (Perez et al., 1997). Complete degradation of different isomers of chlorophenols occurred in N-deficient static cultures in the presence of glucose and ammonium tartrate. The chlorophenol degradation rate decreases when ammonium tartrate was replaced by ammonium chloride.

7.8 PHYSIOLOGICAL ALTERATIONS OF FUNGI BY PHENOLS

Certain physiological alterations by phenolic substrates are found in certain Micromycetes, such as changes in fructification and abnormal production of pigments or viscous substances (Rahouti et al., 1999). Nearly 50 to 68% of these fungi produced normal fructifications on catechol, protocatechuic, vanillic, ferulic, and syringic acids. The lowest number of fungi was associated with phenol (29%) and guaiacol (40%), respectively. Catechol, guaiacol, and syringic acid induced the strongest pigments. Pigment formation was infrequent with ferulic, vanillic, and protocatechuic acids. *Sporormiella antarctica* and *Heterobasidion annosum* produced abnormal pigments on phenol. Pigment formation was related to the action of phenol oxidases, which polymerize phenols or form quinones. In disubstituted phenols, pigment production was higher with a methoxylated compound such as guaiacol than with catechol.

Among *para*-hydroxybenzoic acids, dimethoxylated syringic acid supports higher pigment production than monomethoxylated vanillic acid and nonmethoxylated protocatechuic acid (Rahouti et al., 1999). About 4.4% of strains secrete viscous substances in the presence of protocatechuic acid. A few strains produce viscous substances in the presence of guaiacol, ferulic, or syringic acids. The significance of production of viscous substances is still unknown. Nearly 8.6% of the strains have been shown to undergo morphological abnormalities, especially among the genera *Alternaria*, *Cylindrocarpon*, and *Fusarium* (Krivobok et al., 1994). Only 0.5% of the strains of the genus *Aspergillus* was discolored or produced a pigment. None of the fructification occurred in the presence of phenol among the Zygomycetes, which normally demonstrate sexual reproduction. Furthermore, the fructifications were more sensitive than the mitotic spores. An inhibitory effect appeared during the formation of fructification in species of *Pleurotus* by incorporation of phenol (0.1 to 1.0 mM) in malt extract agar medium (Upadhyay and Hofrichter, 1993). Soaking wheat straw up to 1600 mg/l showed better mycelial growth and fructification of *Pleurotus cornucopiae*, *P. ostreatus* Z-15, and *Calocybe indica* than those of water-soaked wheat straw.

7.9   TAXONOMIC RELATIONSHIP OF PHENOL-UTILIZING YEASTS AND FUNGI

The distribution of 1044 strains and species of fungi was shown to be determined by their good or medium growth on seven phenolic compounds (Rahouti et al., 1999). The highest percentage of these fungi is classified among Melanconiales, Mucedinaceae, Tuberculariales, and Sphaeropsidales of Fungi Imperfecti. Melanconiales and Ascomycetes show better growth on phenolic acids than on other phenols. Tuberculariales perform well on all phenolics, especially on phenolic acids. A few yeasts grow on phenolic compounds. The taxonomic relevance among heterobasidiomycetous yeasts has been correlated based on the capacity to assimilate 20 low-molecular-weight aromatic compounds by 332 strains representing about 200 species (Sampaio, 1999). Protocatechuic, caffeic, and *para*-hydroxybenzoic acids were mostly assimilated, whereas cinnamic, sinapic, and syringic aids and guaiacol were never utilized. Among the Urediniomycetes, the members of the Sporidiales and Naohidea-Rhodotorula minuta clade display a good ability to assimilate aromatic compounds, whereas the members of the Agaricostilbum-Kondoa group are more heterogeneous, showing similarity with the four subclades known. Among the Tremellomycetidae, the members of the Cystofilobasidium and Tremella clades exhibit less or no ability to utilize aromatic compounds. On the contrary, the members of the Trichosporon clade utilize phenol and similar substrates, and those of Filobasidium clade assimilate various aromatic compounds, including those requiring more complex catabolic routes.

Various taxonomic groups of 809 strains of Micromycetes were shown to be subject to response to consumption of phenol (Krivobok et al., 1994). Five groups of activity were classified based on the criteria of growth. The most efficient strains belonged to the Zygomycetes, followed by Basidiomycetes, Ascomycetes, Coelomycetes, and Tuberculariales. Taxonomically, Mucedinaceae (genus *Penicillium*) assimilated phenol highly, followed by Tuberculariales and Zygomycetes. *Penicillium* and *Fusarium* performed the best and better, respectively, while the *Aspergilli* behaved poorly, as did the other Mucedinaceae. On the contrary, the yeasts (90%), Mucedinaceae (69%), excluding *Aspergillus* and *Penicillium*, and Dematiaceae (66%), were the least efficient for phenol.

During screening of 784 strains of Deuteromycetes, five classes of activity were divided into different taxonomic groups based on PCP depletion: 0–20, 20–40, 40–60, 60–80, and more than 80% (Seigle-Murandi et al., 1995). The efficient strains showed high heterogeneity, depending on the groups and genera. Among the most efficient fungi, 168 revealed 70% PCP depletion and 48 showed 80% (Mucedinaceae, 23; Dematiaceae, 14; Sphaeropsidales, 2; Tuberculariales, 1). *Aspergillus* and *Penicillium* were the most active and were considered separately from other Mucedinaceae, due to the production of higher levels of phenol oxidase than others. Ellis (1971) classified *Aspergil-*

lus niger with Dematiaceae, but high levels of phenol oxidase production did not fit in this group. The same confusing observation was made for Ascomycetes. Yeasts showed no appetite for PCP and their inability to produce phenol oxidase, whereas Basidiomycetes depleted PCP moderately along with a high production of phenol oxidase (Steiman et al., 1994). However, Zygomycetes showed the homogeneous behavior of extensive depletion of PCP without any phenol oxidase activity (Seigle-Murandi et al., 1992). Spores of strains of the same taxonomic group exhibited a difference in unknown PCP sensitivity.

It is almost impossible to classify genera or species of yeasts and fungi on the basis of the extent of degradation or degradation capacities. However, the elucidation of metabolic pathways, secretion of enzymes, and genetic studies may provide good insight into the taxonomic relationship of phenol-utilizing yeasts and fungi in the future.

7.10   MECHANISMS OF METABOLISM, METABOLIC PATHWAYS, AND METABOLITES

Aerobic degradation of phenol by bacteria has been examined since the beginning of the twentieth century. Several aerobic phenol-degrading bacteria have been isolated, and pathways for the degradation of phenol were initiated in 1950 and have now been well described. Van Schie and Young (2000) described the aerobic pathways, and phenol hydroxylase enzymes and their genetic analysis, anaerobic biodegradation, and phenol carboxylation under denitrifying and methanogenic conditions in bacteria.

The biodegradation of phenol by fungi started in 1960s, and the pathways began to be clarified in the 1970s. However, most of the work on the pathways of degradation of phenol and its derivatives has focused on yeasts, species of *Aspergillus* and *Penicillium*, and white-rot fungi. This includes the involvement of oxygen and cleavage of the benzene nucleus. The metabolism of phenol and its derivatives by yeasts (Neujahr, 1990) and of phenols, chlorophenols, and PCP by fungi (Kremer and Anke, 1997) has been described. Table 7.5 lists rates of degradation or mineralization, metabolic products, and pathways of various phenolics, chlorophenols, and pentachlorophenol by fungi.

Scedosporium apiospermum, a hyphomycete isolated from the soil, effectively degraded both phenol and *para*-cresol as the sole sources of carbon and energy at pH 7.3 and 37°C (Clauβen and Schmidt, 1998). All *ortho*-type ring-cleaving dioxygenases were detected. The catabolism of *para*-cresol followed a single pathway to yield 3-oxoadipate, and the entire catabolism of phenol followed two different enzymatic sequences. Two possible routes in the catabolism of phenol by *S. apiospermum* are outlined in Figure 7.3. Enzyme activities and the corresponding metabolites of two different catabolic routes have been detected. In one pathway, the catabolism of phenol is followed by

TABLE 7.5 Fungal Metabolism of Phenols and Metabolic Products

Fungus	Phenolic(s) Conc.	Medium	Degradation/ Mineralization Rate (%)	Metabolic Products	Pathway	Duration (hours/days)	Reference
Aspergillus fumigatus	Phenol, 2.1 mM	Liquid medium	>99	Catechol, hydroquinone	2	12 h	Jones et al., 1995
A. fumigatus	4-Ethylphenol, 1.6 mM	Liquid medium	>99	1-(4'-Hydroxyphenyl)ethanol, 4-hydroxyacetophenone, hydroquinone	1	12 h	Jones et al., 1994
Penicillium simplicissimum	Phenol, 8.5 mM	Mineral salt medium	Mineralized	Catechol, hydroquinone, *cis,cis*-muconic acid			Marr et al., 1996
	3-Chlorophenol, 0.5 mM		83	4-Chlorocatechol, chlorohydroquinone, 4-chloro-1,2,3-trihydroxybenzene, 5-chloro-1,2,3-trihydroxybenzene	1	22 d	
	4-Chlorophenol, 0.5 mM and 1 mM phenol		100	4-Chlorocatechol	1	8 d	
	4-Bromophenol, 0.5 mM and 1 mM phenol		90			28 d	
	2-Fluorophenol, 0.5 mM and 1 mM phenol		100			4 d	
	3-Fluorophenol, 0.5 mM and 1 mM phenol		100			13 d	
	4-Fluorophenol, 0.5 mM and 1 mM phenol		100			7 d	

Organism	Substrate	Medium	%	Products	No.	Time	Reference
Penicillium frequentans Bi 7/2	4-Fluorophenol, 2 mM	Potassium-phosphate medium (phenol-grown mycelia)	100	4-Fluorocatechol, 4-carboxymethylenebut-2-en-4-olide	1	18 h	Hofrichter et al., 1994
	3-Fluorophenol, 2 mM		100	4-Fluorocatechol, 3-fluorocatechol, 4-carboxymethylenebut-2-en-4-olide, 2-fluoromuconic acid		25 h	
	2-Fluorophenol, 2 mM		100	3-Fluorocatechol, 2-fluoromuconic acid		55 h	
	4-Chlorophenol, 1.4 mM		100	4-Chlorocatechol, 4-carboxymethylenebut-2-en-4-olide	1	48 h	
	3-Chlorophenol, 1.0 mM		100	4-Chlorocatechol, 3-chlorocatechol, 4-carboxymethylenebut-2-en-4-olide		37 h	
	2-Chlorophenol, 0.75 mM		100	3-Chlorocatechol		180 h	
	3,4-Dichlorophenol, 30 mg/l		100	4,5-Dichlorocatechol	1	75 h	
	2,4-Dichlorophenol, 30 mg/l	Czapek–Dox medium with 0.5 g/l phenol	82	3,5-Dichlorocatechol	1	180 h	
Scedosporium apiospermum	Phenol, 5 mM	Mineral salts medium		Hydroquinone	2	5 d	Claußen and Schmidt, 1998, 1999
	p-Cresol, 5 mM			4-Hydroxybenzoate, 4-hydroxybenzyl alcohol	1		
	Phenylbenzoate			Benzoate and phenol	1		
	p-Tolylbenzoate			Benzoate, *p*-cresol	1		
	4-Chlorobenzoate each 0.5–5 mM			Benzoate, 4-chlorophenol			

TABLE 7.5 Continued

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Fungus	Phenolic(s) Conc.	Medium	Degradation/ Mineralization Rate (%)	Metabolic Products	Pathway	Duration (hours/days)	Reference
Brettanomyces anomalus	Caffeic acid, 2 mM Ferulic acid, 2 mM *p*-Coumaric acid, 2 mM Vanillin, 2 mM	Defined medium		Vinyl and ethyl catechols Vinyl and ethyl guaiacols Vinyl and ethyl phenols Vanillyl alcohol, vanillic acid		200 h	Edlin et al., 1995
Candida aquaetextoris	4-(1-Nonyl)phenol, 100 mg/l	Yeast nitrogen base broth	>99	*trans*-4-Hydroxycinnamic acid, 4-hydroxyacetophenone	1	14 d	Vallini et al., 2001
Trametes versicolor	2-CP, 500 μM	Whole fungal culture	100	2-Chloro-1,4-benzoquinone		3 d	Grey et al., 1998
		Fungal mycelia Cell-free crude culture liquids	100 97	2-Chloro-1,4-benzoquinone 2-Chloro-1,4-benzoquinone		68 h 70 h	
Candida maltosa	3-,4-CP, 0.6 mM each	Mineral salt Phenol-induced cells	100 100	3- and 4-Chlorocatechol, 5-chloropyrogallol, 4-carboxymethylenebut-2-en-4-olide		5 h 5 h	Polnisch et al., 1992
	2-CP, 0.4 mM		75	3-Chlorocatechol, *cis*, *cis*-2-chloromuconic acid		25 h	
Phanerochaete chrysosporium	3,4-Dichlorophenol	C-limited medium	30	3,4-Dichloroanisole, 4,5-dichloroguaiacol, 4,5-dichloroveratrole			Deschler et al., 1998
Mycena avenacea	PCP, 100 mg/l	YMG medium	89	2,3,5,6-Tetrachloro-*p*-benzoquinone, 2,3,5,6-tetrachloro-*p*-hydroquinone, 3,5, 6-trichloro-2-hydroxy-*p*-benzoquinone	1	9 d	Kremer et al., 1992
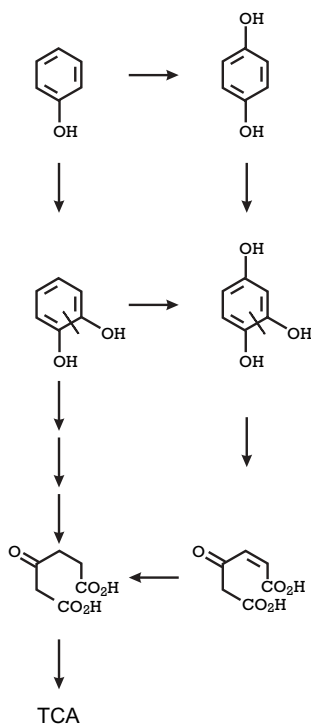
Figure 7.3 Metabolism of phenol via two routes by *Scedosporium apiospermum*. See the text for the identifying compounds. [Reprinted from Claußen and Schmidt (1998), copyright © with permission from Elsevier.]

catechol and 3-oxoadipate, and the second pathway goes to hydroquinone, 1,2,4-trihydroxybenzene, maleylacetate, and finally, to 3-oxoadipate. Phenyl-benzoate hydrolyzes in mineral salts medium to yield phenol and benzoate by inducible esterase using *Scedosporium apiospermum* (Claußen and Schmidt, 1999). Enzymes are induced for both the catabolism of benzoate via the protocatechuate *ortho*-pathway and the degradation of phenol via 3-oxoadipate. Phenol is catabolized via the known routes of catechol and hydro-quinone, and benzoate is degraded via the protocatechuate branch of the *ortho*-pathway. Evidence of the operative gentisate pathway exists for the degradation of some phenols and hydroxybenzoates by the Imperfect ascomycetous yeasts *Candida parapsilosis* and *Arxula adeninivorans* (Middelhoven et al., 1992b). 4-Hydroxybenzoic acid degrades via protocate-chuate. No cleavage enzyme has been detected, but the NADH- and NADPH-dependent monooxygenases are present. *A. adeninivorans* shows glutathione-dependent isomerization of maleylpyruvate to fumarylpyruvate in the metabolism of 3-hydroxybenzoate.

Phenol is utilized as a sole source of carbon and energy by the thermotoler-ant strain of *Aspergillus fumigatus* ATCC 28282 (Jones et al., 1995).
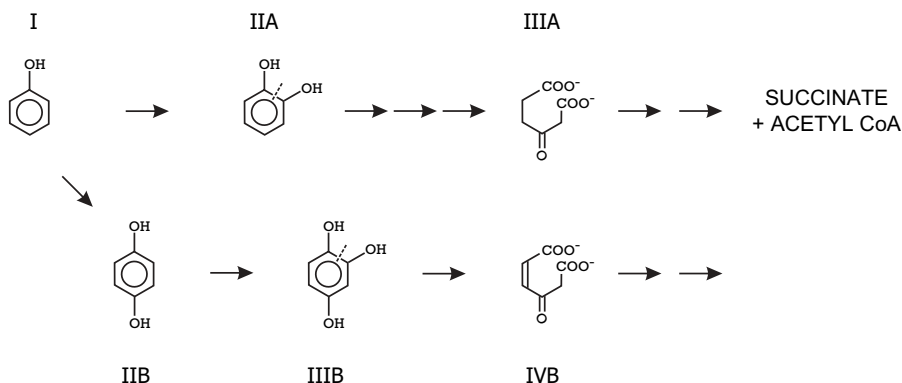
Figure 7.4 Pathways for metabolism of phenol by *Aspergillus fumigatus* ATCC 28282. I, phenol; IIA, catechol; IIIA, 3-oxoadipate; IIB, hydroquinone; IIIB, 1,2,4-trihydroxybenzene; IVB, maleylacetate. [Reprinted from Jones et al. (1995), copyright © with permission from Springer Science and Business Media and the authors.]

Metabolism of phenol occurs via two possible routes, leading to different ring fission substrates (Figure 7.4). In one route, phenol hydroxylates at the *ortho*-position to produce catechol, which is cleaved to 3-oxoadipate by an intradiol mechanism. In another route, phenol hydroxylates at the *para*-position to form hydroquinone, which is converted to 1,2,4-trihydroxybenzene and subsequently, a ring is cleaved to produce maleylacetate. The same fungal strain also assimilates 4-ethylphenol (Jones et al., 1994) and *para*-cresol (Jones et al., 1993) as the sole carbon sources. A pathway has also been proposed for the metabolism of 4-ethylphenol by *A. fumigatus*. The metabolism of 4-ethylphenol by *A. fumigatus* follows a common sequence of metabolites to hydroquinone established for *Pseudomonas putida* JD1 (Darby et al., 1987). However, during the initial step, the bacterium uses a flavocytochrome *c* dehydrogenase–hydratase and the fungus a monooxygenase. Jones et al. (1993) also demonstrated the conversion of *para*-cresol into protocatechuate by two routes using *A. fumigatus*, and the difference is in the sequence of reactions. *Penicillium simplicissimum* SK9117 can utilize phenol and fluorinated phenols as the sole sources of carbon and energy (Marr et al., 1996). The metabolism of all monofluorophenols is enhanced in the presence of phenol, resulting in the simultanous disappearance of substrate and cosubstrate. The equimolar release of chloride ions indicates complete mineralization of 3- and 4-fluorophenols. Based on the metabolites identified, a pathway is proposed for the metabolism of monochlorophenols. Resting phenol-grown mycelia of *Penicillium frequentans* Bi 7/2 were shown to degrade the monochlorinated phenols, 3,4-dichlorophenol, and some other halogenated phenols completely within different time intervals (Hofrichter et al., 1994). Pathways for the degradation of monochlorinated derivatives by *P. frequentans* Bi 7/2 were proposed.

Two main metabolites, *trans*-4-hydroxycinnamic acid and 4-hydroxyaceto-
phenone (4-acetylphenol), were identified during the metabolism of 4-
(1-nonyl)phenol (*p*NP) by *Candida aquaetextoris* (Vallini et al., 2001).
4-Acetylphenol accumulated in the culture without further degradation.
However, traces of an unidentified *para*-substituted phenol were detected.
Good growth of *C. aquaetextoris* on 3-(4-hydroxyphenyl)-propionic acid also
produced both *trans*-4-hydroxycinnamic acid and 4-hydroxyacetophenone.
Poor growth and no evidence of growth were obtained on *trans*-4-hydroxycin-
namic acid and 4-hydroxyacetophenone, respectively. Biodegradation of *p*NP
occurred via terminal oxidation of the alkyl chain, followed by a β-oxidation
pathway. A novel metabolic route for the degradation of *p*NP in certain yeasts
has also been proposed. Corti et al. (1995) identified 4-hydroxyacetophenone
as one of the metabolites from *p*NP by a strain of *Candida* yeast that was later
classified as *C. aquaetextoris* (Vallini et al., 1997).

4-Hydroxycinnamic acids are carboxylated and subsequently reduced to
their 4-vinyl and 4-ethyl derivatives by the yeasts *Brettanomyces intermedius*
and *B. anomalus* (Heresztyn, 1986; Lauritsen et al., 1991; Chattonet et al.,
1992). *Brettanomyces anomalus* NCYC 615 transforms *para*-coumaric acid,
caffeic acid, ferulic acid, and vanillin to yield two major products due to C2
cleavage (Edlin et al., 1995). *B. intermedius* also has the ability to metabolize
para-coumaric, ferulic, and sinapic acids in a similar way in unshaken flasks
(Heresztyn, 1986). Sinapic acid is not metabolized by *B. anomalus*. Two
enzymes catalyze the metabolism of 4-hydroxycinnamic acids by *Brettano-
myces* yeasts (Figure 7.5). Cinnamic decarboxylase is the first enzyme respon-
sible for the cleavage of C3 carbon from the side chain, releasing CO_2. Vinyl
phenol reductase is the second enzyme that reduces the double bond on the
vinyl derivative to form ethyl-substituted compounds. The differences in the
specificity of cinnamic hydroxylase result in metabolic differences of hydroxy-
cinnamic acids by two species of yeasts. *B. anomalus* transforms vanillin to
vanillyl alcohol and vanillic acid (Figure 7.6). De Wulf et al. (1986) reported
similar results in *Saccharomyces cerevisiae*. These reactions involve oxido-
reductase enzymes and require an electron donor–acceptor such as

R = H, OH, CH₃O
CD = Cinnamate Decarboxylase
VPR = Vinyl Phenol Reductase
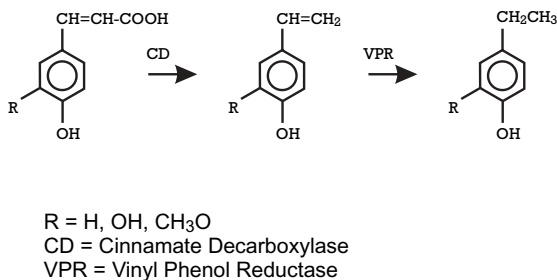
Figure 7.5 Metabolism of 4-hydroxycinnamic acids by *Brettanomyces anomalus*.
[Reprinted from Edlin et al. (1995), copyright © with permission from Elsevier.]
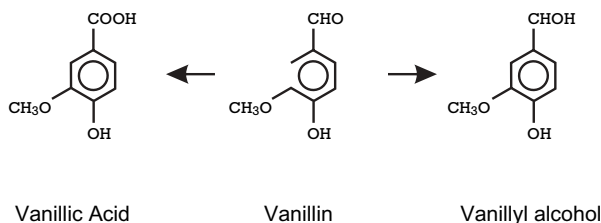
Figure 7.6 Conversion of vanillin to vanillyl alcohol and vanillic acid by *Brettanomyces anomalus*. [Reprinted from Edlin et al. (1995), copyright © with permission from Elsevier.]

nicotinamide or flavin adenine dinucleotide/nicotinamide or flavin adenine dinucleotide phosphate (NAD/NADH) or (FAD/FADH). Transformation of *para*-coumaric acid by *Curvularia lunata* showed the formation of several intermediates (Torres and Rosazza, 2001). However, the presence of peroxidase and laccase activities indicated the involvement in one-electron dimerization.

The formation of only the metabolite 2-chloro-1,4-benzoquinone (2-ClBQ) was detected during degradation of 2-CP by whole *Trametes versicolor* cultures, fungal mycelia, and cell-free crude culture liquids (Grey et al., 1998). The production of 2-ClBQ is correlated with the extracellular laccase activity in cell-free crude culture liquids. On the contrary, the disappearance of 2-CP by the whole fungus does not correlate with extracellular laccase activity. The extracellular laccase can be partially responsible for the oxidative step, indicating involvement of a cell-bound process. Minard et al. (1981) also reported the formation of 2-ClBQ during the oxidation of 2-dichlorophenol by laccase from *Rhizoctonia praticola* and by LiP and MnP that catalyze one-electron abstractions from phenolic compounds. Valli and Gold (1991) also detected 2-ClBQ during the degradation of 2-dichlorophenol by *Phanerochaete chrysosporium*. Methylation and hydroxylation reactions are involved in the degradation of 3,4-dichlorophenol (3,4-DCP) by *P. chrysosporium*, followed by the formation of three metabolites (Deschler et al., 1998). Phenolics and benzoic acid derivatives are utilized as the sole sources of carbon by fungi such as *Chaetomium cupreum*, *Drechslera oryzae*, and *Fusarium oxysporum* f.sp. *vasinfectum* (Boominathan and Mahadevan, 1989). The central metabolites isolated by replacement culture are catechol, protocatechuic acid, hydroxyquinol, gentisic acid, and homogentisic acid. The fungi exhibit catechol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase, hydroxyquinol 1,2-dioxygenase, gentisate oxygenase, and homogentisate oxygenase during growth on these aromatics. Pathways for the degradation of these aromatics have been proposed. The 3-oxoadipate pathway of catechol and protocatechuate and ring fission of 1,2,4-trihydroxybenzene in fungi have also been proposed (Cain, 1987). Neujahr (1990) described the role of oxygen in the degradation of phenolic compounds, general pathways

(*ortho-* and *meta-*fission) of metabolism of phenols, and cleavage of benzene nucleus by yeasts, including the metabolic versatility of *Trichosporon cutaneum*.

7.11   DEGRADATION OF PHENOLS BY FUNGAL ENZYMES

Most phenols are toxic and produce coloration to receiving waters and thus must be removed. Conventional methods of dephenolization of wastewaters have certain drawbacks, such as high cost, incomplete purification, or restricted applicability. Biological methods are of interest due to low cost and the low adverse effect on the environment. Due to their biocide nature, most phenols cannot be degraded in conventional biological wastewater treatment, and the presence of high concentration of phenols may spoil the live microorganisms in the system.

Enzyme treatment opens up a new approach for the treatment of wastewaters containing hazardous phenolic compounds. The use of enzymes allows the process of degradation under mild biological conditions. Phenols can be oxidized by certain enzymes employing either hydrogen peroxide or molecular oxygen–producing phenoxy radicals that react by themselves or with other phenols to form dimers. These reactions lead to the formation of precipitates of higher oligomers and polymers of low solubility and can be removed easily by sedimentation or filtration. There are some potential advantages of enzymatic treatment compared to conventional treatment (Nicell et al., 1993): applicability at high and low contaminant concentrations over a wide range of pH, temperature, and salinity; applicability to biorefractory compounds; unamenability to loading effects; no acclimatization of biomass; and reduction in sludge volume and ease of controlling the process.

The free or immobilized preparation of enzymes can be applied to degrade or remove compounds that are unable to be treated by whole cell systems. The use of peroxidase, tyrosinase, or laccase enzymes in the detoxification of phenols and aromatic amines from drinking water and wastewater was started in 1980s, and our knowledge is still far from complete. In recent years, considerable attention has been given to immobilized enzymes, which act as biocatalysts in the degradation of toxic organic compounds. The use of soluble enzyme is impractical due to the requirement for a large quantity and harsh denaturing conditions in the system. Such drawbacks can be eliminated by immobilizing enzymes on a solid support system. Siddique et al. (1993) tested immobilized enzymes for the degradation of 4-chlorophenol from aqueous solution. Co-immobilized cell mass and enzyme from *Phanerochaete chrysosporium* were employed for the degradation of PCP (Lin et al., 1991). The potential applications of enzymes [horseradish peroxide (HRP), lignin peroxidase (LiP), manganese peroxidase (MnP), and other peroxidases, tyrosine, and laccases related to phenolic contaminants] have been described by Karam and Nicell (1997).

7.11.1 Peroxidase-Catalyzed Degradation

Lignin peroxidase (LiP) is a fungal heme protein and oxidizes dimethylated aromatic compounds to radical cations. Paszczynski et al. (1986) first reported an extensive study on the oxidation of phenols by LiP. A mechanism for the veratryl alcohol–mediated indirect oxidation of phenol by LiP has been proposed (Chung and Aust, 1995). Table 7.6 lists rates of degradation or mineralization, metabolic products of phenolics, chlorophenols, and PCP by fungal peroxidases.

Only about 10% of PCP remains in nitrogen-limited cultures after 30 hours by *P. chrysosporium* (Reddy and Gold, 2000). However, about 90% of PCP remains under nitrogen-sufficient conditions. Three metabolites are produced in nitrogen-limited cultures. Under nitrogen-sufficient conditions, PCP undergoes *ortho*-methylation to produce pentachloroanisole as a sole metabolite. Several metabolites are identified during the respective addition of tetrachlorodihydroxybenzene, trichlorodihydroxybenzene, and 2,5-dichloro-1,4-dihydroxybenzene in nitrogen-limited cultures. Similar metabolites are identified during transformation of these compounds in nitrogen-sufficient cultures. LiP and MnP catalyze oxidations of PCP to chlorobenzoquinone. Both enzymes also oxidize tetrachlorodihydroxybenzene to its corresponding benzoquinone. A pathway proposed for the complete dechlorination of PCP by *P. chrysosporium* is shown in Figure 7.7. Reductive dechlorination reactions are also involved in the degradation of 2,4,6-TCP by *P. chrysosporium* and several other white-rot fungi (Reddy et al., 1998). A similar quinone reduction step has also been noted during degradation of 2,4,5-TCP and 2,4-DCP by *P. chrysosporium* (Valli and Gold, 1991; Joshi and Gold, 1993).

Four metabolites are generated by withdrawal of one-electron oxidation of bisphenol A (BPA) by MnP of *Pleurotus ostreatus* (Hirano et al., 2000). A pathway of BPA metabolism by MnP of *P. ostreatus* has been proposed which is quite different from bacteria (Spivack et al., 1994). BPA is converted to compounds with no estrogen activity by MnP of *P. chrysosporium* ME-466, and the main products are considered to be trimers and tetramers (Tsutsumi et al., 2001). The initial steps of ferulic acid polymerization by LiP isozyme H1 from high-nitrogen cultures of *Phanerochaete chrysosporium* BKM-F-1767 lead to the formation of three dehydrodimers and two trimers (Ward et al., 2001). This is the first report of identification of trimers from LiP oxidation of ferulic acid, and these are formed by decarboxylation of dehydrodimer intermediates. The dehydrodimers and trimers are further oxidized by LiP, implying that they are the only intermediates in the polymerization of ferulic acid.

7.11.1.1   Peroxidase Bioreactors. A two-stage immobilized MnP bioreactor has been optimized for the catalytic generation of Mn(III) and subsequent oxidation of chlorophenols using *Lentinula* (*Lentinus*) *edodes* (Grabski et al., 1998). Purified MnP from *L. edodes* was covalently coupled through its carboxyl groups using an azlactone-functional copolymer of EEDQ

TABLE 7.6 Peroxidase-Catalyzed Degradation of Phenols and Metabolic Products

Fungus	Enzyme Conc.	Phenolic Compound/ Conc.	Medium/ Type of Bioreactor	Degradation/ Mineralization Rate (%)	Metabolic Products/ Pathway	Duration (hours/ minutes)	Reference
Phanerochaete chrysosporium		PCP, 250μM	N-limited	90	Tetrachlorodihydroxybenzene, tetrachlorobenzoquinone, pentachloroanisole	30 h	Reddy and Gold, 2000
		Tetrachloro-dihydroxybenzene, 250 μM	N-sufficient N-limited	10 80	Pentachloroanisole 2,3,5-Trichloro-1, 4-dihydroxybenzene, trichloro-1,2, 4-trihydroxybenzene, dichlorotrihydroxybenzene, tetrachloro- 4-methoxyphenol, tetrachloro-1, 4-dimethoxybenzene, tetrachlorobenzoquinone	30 h	
		Trichloro-dihydroxybenzene, 250 μM	N-sufficient N-limited	100	Similar products 2,5-Dichloro-1, 4-dihydroxybenzene, 3,5,6-trichloro-1,2, 4-trihydroxybenzene, dichlorotrihydroxybenzene, 2,3,5-trichloro- 4-methoxyphenol, 2,3,5-trichloro-1, 4-dimethoxybenzene		
		2,5-Dichloro-1, 4-dihydroxybenzene, 250 μM	N-sufficient N-limited		Similar products 2-Chloro-1, 4-dihydroxybenzene, 5-chloro-1,2, 4-trihydroxybenzene, 1,2,4-trihydroxybenzene, 2,5-dichloro-1,3, 4-trihydroxybenzene, 2,5-dichloro-1, 4-dimethoxybenzene, 2,5-dichloro- 4-methoxyphenol		

TABLE 7.6 Continued

Fungus	Enzyme Conc.	Phenolic Compound/ Conc.	Medium/ Type of Bioreactor	Degradation/ Mineralization Rate (%)	Metabolic Products/ Pathway	Duration (hours/ minutes)	Reference
	LiP and MnP LiP and MnP	PCP Tetrachloro-dihydroxybenzene	N-sufficient		Similar products Tetrachlorobenzoquinone Benzoquinone Known pathway	1h	Hirano et al., 2000
Pleurotus ostreatus	MnP 20U	Bisphenol A, 0.4mM	Sodium lactate buffer		Phenol, 4-isopropenylphenol, 4-isopropylphenol, hexestrol Known pathway		Tsutsumi et al., 2001
P. chrysosporium ME-466	MnP	Bisphenol A			Trimers and tetramers		
Lentinula edodes	MnP, laccase, and β-glucosidase (660-kDa protein)	PCP, 50μM 2,5-DCP, 50μM	McIllvaine buffer	52 23	Unknown pathway	16h	Makkar et al., 2001
P. chrysosporium BKM-F-1767	LiP-H1, 1 μM and H_2O_2 (various amounts)	Ferulic acid 300μM	Sodium tartrate buffer		Dehydrodimers and trimers Partial pathway	30m	Ward et al., 2001
L. edodes	MnP, 5 mg/ml	2,4-DCP 2,4,6-TCP	Reactor 1 immobilized with EEDQ Emphaze beads and reactor 2 of oxidation reaction		Unidentified products Unidentified products Unknown pathway	24h	Grabski et al., 1998
Coprinus macrorhizus	CMP, 0.6 U/ml	Phenol, 1 mM	Batch reactor (buffer, H_2O_2) Continuous reactor (buffer, H_2O_2)	53 91	 Unknown pathway	<2h 3h	Al-Kassim et al., 1994a
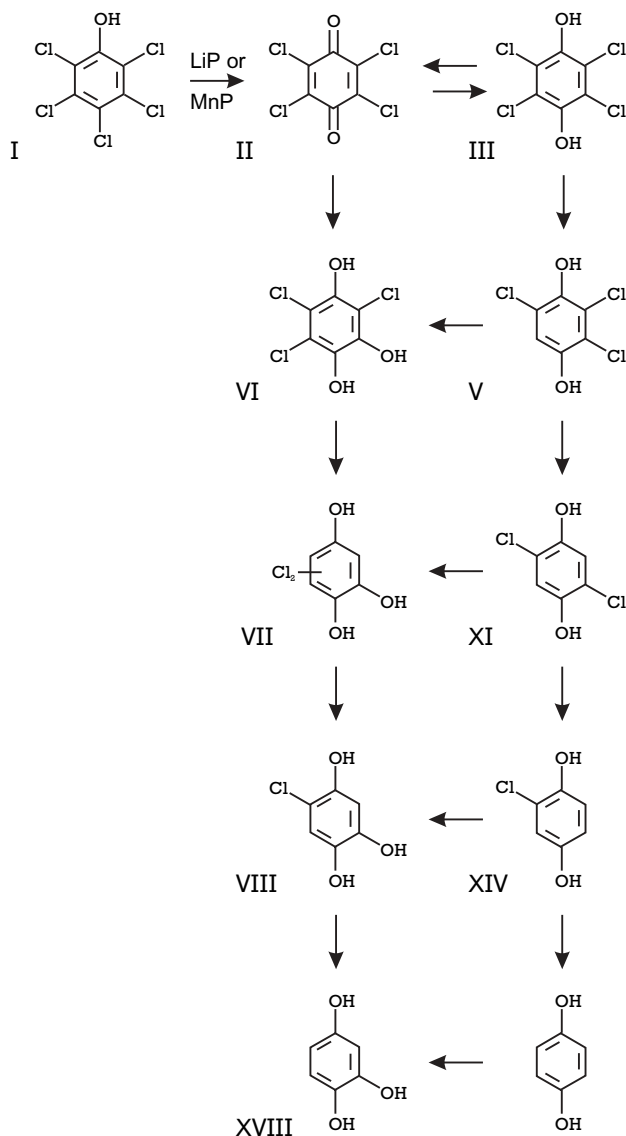
Figure 7.7 Tentative pathways for the degradation of PCP (I) by *Phanerochaete chrysosporium*. II, tetrachlorobenzoquinone; III, tetrachlorodihydroxybenzene; V, trichlorodihydroxybenzene; VI, trichlorotrihydroxybenzene; VII, dichlorotrihydroxybenzene; VIII, monochlorotrihydroxybenzene; XI, dichlorodihydroxybenzene; XIV, monochlorodihydroxybenzene; XVIII, trihydroxybenzene. [Reprinted from Reddy and Gold (2000), copyright © with permission from the Society for General Microbiology.]
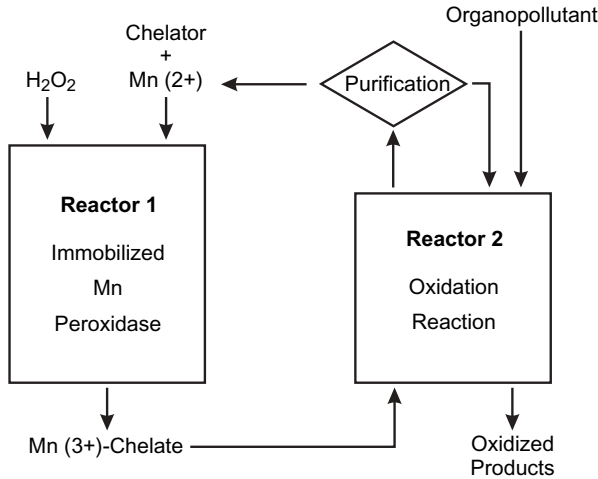
Figure 7.8   Two-stage immobilized MnP oxidation reactor for enzymatic generation of Mn(III)-chelates by reactor 1, and subsequent oxidation of chlorophenols in reactor 2. [Reprinted from Grabski et al. (1998), copyright © with permission from John Wiley & Sons.]

(ethylenediamine and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline) Emphaze beads. Figure 7.8 shows MnP immobilized reactor 1, catalyzing the H_2O_2-dependent oxidation of Mn(II), generating Mn(III)-chelates. Mn(III)-chelates are pumped to reactor 2 for the oxidation of chlorophenols, and the oxidation products along with Mn(II) can be removed or returned to reactor 1 for regeneration. The optimized conditions resulted in oxidation of 1.0 mM Mn(II) to Mn(III) at 78% efficiency and about 69% efficiency after 24 hours of continuous operation.

Phenol can be removed from synthetic wastewater in batch, continuous, and discontinuous semibatch reactors by *Coprinus macrorhizus* peroxidase (CMP) (Al-Kassim et al., 1994a). Nearly 90% phenol was eliminated with the addition of three discrete aliquots of either H_2O_2 or CMP over 0.5 hour in discontinuous reactors. Discontinuous addition of the reactants enhanced the removal of phenol by CMP and conserved 1:1 phenol to peroxide stoichiometry in the reaction. Al-Kassim et al. (1994b) also reported catalysis of the same reactions by *C. macrorhizus* peroxidase (CMP) as HRP in the removal of toxic organics from synthetic wastewater. Kjalke et al. (1992) reported the similarity of *Arthromyces ramosus* peroxidase (ARP) to *Coprinus cinereus* peroxidase (CIP) and *C. macrorhizus* peroxidase (CMP) and a close taxonomic relationship between *Coprinus* and *Arthromyces* species.

7.11.2   Polyphenol Oxidase/Tyrosinase–Catalyzed Degradation

Polyphenol oxidase (PPO) or tyrosinase is a copper enzyme widely distributed in plants and fungi. The structural and sequential aspects of fungal

Phenol $\xrightarrow{O_2}$ Catechol $\xrightarrow{O_2}$ o-Quinone
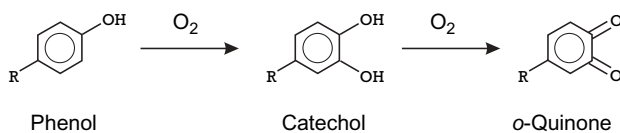
Figure 7.9 Polyphenol oxidase–mediated conversion of phenols to o-quinones.

tyrosinases have been discussed by van Gelder et al. (1997). PPO can be obtained from fungal sources such as the common mushroom *Agaricus bisporus* and the bread mold *Neurospora crassa*. PPO catalyzes the hydroxylation of monophenols to *ortho*-diphenols using O_2 to yield catechols and subsequent oxidation to form *ortho*-quinones (Figure 7.9). The *ortho*-quinones undergo spontaneous nonenzymatic polymerization producing water-insoluble polymers that can be separated from solution by filtration. PPO requires O_2 as an oxidant compared to stoichiometric amounts of H_2O_2 by peroxidase. The major drawback of PPO is the marked product inhibition caused by *ortho*-quinones binding with the active site of the enzyme. PPO can be applied as a waste minimization strategy for effluent treatment. Dec and Bollag (1990) examined the ability of mushroom tyrosinase to polymerize substituted phenols. The optimum pH for tyrosinase ranged from 4.5 to 8.5 and the optimum temperature was 25°C. Removal of dehalogenated phenols decreased with an increasing number of chlorines and an increasing molecular weight of the substituent.

The kinetics between 4-*tert*-butylphenol (4-TBP) and mushroom tyrosinase has been studied by Ros et al. (1994). The kinetics of oxidation of phenol substrates by tyrosinase has been widely discussed (Naish-Byfield et al., 1994; Naish-Byfield and Riley, 1998). Such studies on the action of PPO to monophenols have been shown to produce fairly stable *ortho*-quinones, such as 4-hydroxyanisole (4-HA), 4-ethoxyphenol (4-EP), and 4-*tert*-butylphenol (4-TBP). A kinetic mechanism based on structural aspects developed by Wilcox et al. (1985) has been proposed to explain enzyme action in Scheme 1a + b (Cabanes et al., 1987). A kinetic mechanism of the direct action of tyrosinase on monophenol releasing *ortho*-diphenol to the reaction medium has been examined by Fenoll et al. (2000). However, several researchers do not concur with this mechanism (Sayre and Nadkarni, 1994; Cooksey et al., 1998). Reducing agents such as Fe(II), serine, and ascorbic acid accelerated the oxidation of monophenols by tyrosinase from *Aspergillus flavipes* 56003, decreasing the lag time of the reaction (Gukasyan, 2002).

7.11.2.1 Polyphenol Oxidase/Tyrosinase Bioreactors. Various types of polyphenol oxidase bioreactors are recognized for removal of the phenolic compounds (Table 7.7). Phenols are removed in an aqueous solution by soluble and immobilized mushroom tyrosinase (Wada et al., 1993). Soluble tyrosinase removes the substituted phenols in the preferential order catechol > *para*-cresol > *para*-chlorophenol > phenol > *para*-methoxyphenol. A color

TABLE 7.7 Polyphenol Oxidase Bioreactors for Degradation of Phenols

Fungus	Type of Bioreactor/ PPO Conc.	Phenolic Compound/Conc.	Total Phenols Converted (% or μM or mg)	Duration (hours)	Reference
Mushroom	Soluble PPO (20 U/ml)	Phenol, 0.05 mM	100%	2–3	Wada et al., 1993
		p-Chlorophenol, 0.05 mM	100%		
		p-Methoxyphenol, 0.05 mM	100%		
		p-Cresol, 0.05 mM	100%		
		Catechol, 0.05 mM	100%		
	Immobilized PPO (21.5 U/ml) on cation-exchange resin	Phenol, 0.2 mM	100%	2	
Agaricus bisporus	Capillary membrane (IPS 763) using immobilized PPO (140 Units)	p-Cresol, 1 mM	20.6 μM	8	Edwards et al., 1999a
	Capillary membrane (IPS 763) using immobilized PPO (45 Units)	Total phenolics, 4 mM	949 μM	8	
	Capillary membrane (IPS 748) using immobilized PPO (140 Units)	p-Cresol, 1 mM	829 μM	8	
A. bisporus	Immobilization of PPO (143 Units) on chitosan-coated polysulfone capillary membrane	Phenol, 0.0197 mM	1224.4 mg	8	Edwards et al., 1999b
A. bisporus	Immobilized PPO (140 Units) on nylon membranes (IPS 748)	p-Cresol, 1 mM	20.6 μM	8	Burton et al., 1998
	Immobilized PPO (140 Units) on nylon membranes (IPS 763)	p-Cresol, 1 mM	829 μM	8	

change from colorless to dark brown occurs without precipitation. The colored products are removed by chitin and chitosan, and chitosan enhances the reduction rate of phenols. Rapid removal of colored products can occur by a combination of tyrosinase and two synthetic polymers or coagulants and chitosan (Wada et al., 1995). The coagulants are more effective than the chitosan. In one study, total phenol (100%) was removed after 2 hours by treatment with immobilized tyrosinase on cation-exchange resin, and very little loss of activity resulted even after 10 repeated treatments (Wada et al., 1993). Immobilized tyrosinase on magnetite showed good retention activity (80%) and storage stability (Wada et al., 1995). Less coagulant was used for the removal of colored reaction products during immobilized tyrosinase treatment. Tyrosinase immobilized between two chitosan gels has been used for phenol removal as a batch system (Sun and Payne, 1996). In recent years, tyrosinase has been immobilized in membranes for the decomposition of phenol.

Chitosan can be processed into a variety of configurations, such as membranes, fibers, capsules, and beads. Capillary membrane (Edwards et al., 1999a) and chitosan-coated internally skinned polysulfone capillary membrane (Edwards et al., 1999b) bioreactors using immobilized PPO of the common mushroom *Agaricus bisporus* can remove a range of phenols in synthetic and industrial effluents. The advantages of capillary membrane technology for biocatalysis are high surface area/volume ratio and the ability to handle high volume capacities; it can perform a reaction simultaneously with a separate function; there is less susceptibility to process disturbances; and the sterilization requirements are reduced. Using 763 membranes, 100% of 1 mM *para*-cresol was removed over 8 hours, while 40% conversion was achieved using IPS 748 membranes (Edwards et al., 1999a). In a mixed effluent, IPS 763 bioreactors removed phenolics better than did IPS 748 membranes. This is due to higher flux operating in the IPS 763 bioreactor, which results in rapid transport of substrates as well as inhibitory substances in the vicinity of the enzyme. Phenols have been converted by immobilized PPO from *A. bisporus* on nylon membranes under various conditions in a semi-continuous mode (Burton et al., 1998). The disappearance of phenol and the high proportion of quinone products were generated in substrates of low concentration in reactions of PPO immobilized on nylon membranes. IPS 748 membrane results in greater removal of *para*-cresol than does IPS 763 membrane.

7.11.3 Laccase-Catalyzed Degradation

Laccase is a heme protein, an attractive *para*-diphenol oxidase type of biocatalyst, and is widely distributed in fungi (Mayer and Staples, 2002). Laccases utilize molecular oxygen rather than hydrogen peroxide. Laccases are known to be induced by various aromatic compounds in white-rot fungi. Laccases generate phenoxy radicals and semiquinones, followed by spontaneous reactions, resulting in the formation of quinones and several di- and

oligomeric coupling products. The phenolic substrate, type of laccase, and pH influence the formation of the product patterns of various phenolic compounds. Different substrate specificities are established for the laccases from different fungi and different laccase isoforms from the same fungus. A multiplicative mathematical model has been developed for laccase-catalyzed polymerization of 1-naphthol and biokinetic parameters of activation energy (Akta et al., 2001). The reaction rate constant has been evaluated for the first time.

Laccases are known to oxidize several phenolics, chlorophenols, and PCP (Table 7.8). Laccase of *Trametes versicolor* oxidizes PCP, forming benzoquinone and *para-* and *ortho-*chloronils (Konishi and Inoue, 1972). PCP and 2,4,6-TCP have been dehalogenated in the presence of *Trametes* laccase and a peroxidase from *P. chrysosporium* (Lyr, 1963; Hammel and Tardone, 1988). Nearly 89% of 2,4-dichlorophenol was transformed along with 23% dechlorination and two dehalogenation number by *T. versicolor* laccase (Dec and Bollag, 1994). Free radicals of 2-CP, 3-CP, 2,4-DCP, and 2,4,5-TCP can couple to each other in 10 different ways during polymerization. The substituted phenols have been polymerized by laccases from *Rhizoctonia praticola* and *T. versicolor* (Dec and Bollag, 1990). The detoxification of phenols due to polymerization depended on the chemical structure and concentration of the substrate, pH of the reaction, activity of the enzyme, length of incubation, and temperature. The optimum pH for *R. praticola* laccase extended from 4.5 to 9.0, and for *T. versicolor* laccase from 3.0 to 8.0.

The relative activity of the compost extract (crude laccase) of *A. bisporus* in terms of time required for oxidation is in the order 2,6-dimethoxyphenol > guaiacol > phenol > ventryl alcohol > aniline (Trejo-Hernandez et al., 2001). Complete oxidation of 1 and 5 mg/l of phenol took place within 48 and 125 hours, respectively. The optimum oxidation of phenol occurred at pH 7.0 and 25°C. The rate of dephenolization was 1.07 μg/U per hour for the commercial enzyme and 1.28 μg/U per hour for the crude compost extract. The formation of diverse products can be attributed to a radical laccase reaction followed by one-electron withdrawal from BPA from *Trametes villosa* (Fukuda et al., 2001; Uchida et al., 2001). Purified extracellular laccase from a fungus of family Chaetomiaceae detoxified BPA and nonylphenol rapidly in the absence of mediators (Saito et al., 2004). Their estrogenic activities were completely removed within 24 hours. Polyoxometalates have also been found to be an effective promoter of laccase-assisted reactions (Carneiro et al., 2000).

In *Panus tigrinus*, MnP catalyzes the primary attack of 2,4,6-TCP in culture, and in *Coriolus versicolor*, laccase catalyzes it predominantly (Leontievsky et al., 2000). Both enzymes in respective fungi degraded 70 to 90% of 2,4,6-TCP and produced similar metabolites after 24 hours. A different mode of regulation of these enzymes is suggested in two fungi. Blue laccase of *P. tigrinus* is also capable of both polymerizing and depolymerizing soil humic acids in vitro (Zavarzina et al., 2004). Purified laccase from *C.*

TABLE 7.8 Laccase-Catalyzed Degradation of Phenols and Metabolic Products

Fungus	Enzyme Conc.	Phenolic Compound/ Conc.	Medium/ Type of Bioreactor	Degradation/ Mineralization Rate (%)	Metabolic Products	Duration (days/ hours/ minutes)	Reference
Trametes versicolor		PCP, 3.5 µM	Liquid medium	21 as $^{14}CO_2$		17 d	Ricotta et al., 1996
	2.4 U/ml	PCP, 3.5 µM	Liquid medium	16 as $^{14}CO_2$		17 d	
	5 U/ml	PCP, 100 mM	Citrate–phosphate buffer		*p*- and *o*-Chloranils	1 d	
Agaricus bisporus	Aqueous compost extract containing 200 U/ml	Phenol, 1 mg/ml; Phenol, 5 mg/ml	Sodium–acetate buffer	100; 100		48 h; 125 h	Trejo-Hernandez et al., 2001
Coriolus versicolor	330 U/ml	Phenol; 2,4-DCP; 2,4,6-TCP; 2,3,6-TCP; 2,4,5-TCP	Unbuffered conditions using distilled water	81; 92; 100; 93; 90		24 h; 3 h; 3 h; 24 h; 24 h	Kadhim et al., 1999
Trametes villosa	Bisphenol A				4-Isopropenylphenol, 5,5'-bis-[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-biphenyl-2,2'-diol		Fukuda et al., 2001; Uchida et al., 2001
T. versicolor	40 U/ml	2,4-DCP; 4-CP, 9 mM	Universal buffer	89; 29		24 h	Dec and Bollag, 1994
T. versicolor	10^5 U/l	2,4-DCP, 10 mM	Citrate–phosphate buffer	>99		20 h	Dec and Bollag, 1990
Rhizoctonia praticola	150000 U/l	2,4-DCP, 10 mM	Phosphate buffer	>95		20 h	
Panus tigrinus	MnP, 20 µg	2,4,6-TCP, 0.5–5 mM	Sodium–lactate succinate buffer	70–90	2,6-Dichloro-1,4-hydroquinol, 2,6-dichloro-1,4-benzoquinone, oligomeric compound	24 h	Leontievsky et al., 2000

TABLE 7.8 *Continued*

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Fungus	Enzyme Conc.	Phenolic Compound/ Conc.	Medium/ Type of Bioreactor	Degradation/ Mineralization Rate (%)	Metabolic Products	Duration (days/ hours/ minutes)	Reference
C. versicolor	Laccase, 20μg	2,4,6-TCP, 0.5–5mM	Sodium–acetate buffer	70–90	Similar products		Ullah et al., 2000a
C. versicolor	100U	PCP, 25μg/ml PCP, 200μg/ml PCP, 100μg/ml	Buffer	100 60	Polymers with *p*- and *o*-Chloranils	72h 72h	Roper et al., 1995
T. versicolor	15 DMP units and 20mM guaiacol	2,4,5-TCP, 2mM 2,4-DCP, 2mM 4-CP, 2 mM	Citrate–phosphate buffer	85 70 20		1h	Ullah et al., 2000b
C. versicolor	<25U⁻¹ substrate	2,4-DCP, 50ppm PCP, 50ppm	Aqueous medium	100 100		5h 24h	Leontievsky et al., 2001
C. versicolor	Soluble 25μg	2,4,6-TCP, 400ppm	Sodium–acetate buffer	100	2,6-Dichlorobenzoquinone, 2,6-dichlorohydroquinone	3h	
	25μl	2,4,6-TCP	Immobilized to Celite R-637 with glutaraldehyde	100	2,6-Dichlorobenzoquinone, 2,6-dichlorohydroquinone, 3,5-dichlorocatechol	30m	
Pleurotus ostreatus	23.4U/g	2,6-Dimethoxyphenol, 1 mM	Immobilized to Eupergit C packed-bed		Brown-reddish precipitates	3h	Hublik and Schinner, 2000
Pleurotus eryngii	300mU/ml 1mM ABTS or HBT	2,4-DCP, 50μM	Sodium–acetate buffer	95		6h	Rodriguez et al., 2004
Pyricularia oryzae	9.8U/cm²	2,4-DCP 2,6-Dimethoxyphenol 2-, 3-, 4-Chlorophenol, 0.1mg/ml Guaiacol, 0.1 mg/ml *o*-, *m*-, *p*-Cresol, 0.1mg/ml α-Naphthol, 0.1 mg/ml	Immobilized in a spiral membrane bioreactor	68 69 47, 38, 43 40 39, 43, 49 56			Lante et al., 2000

versicolor eliminated 100% and 60% of 25 μg/ml and 200 μg/ml PCP, respectively, within 72 hours at pH 5.0 (Ullah et al., 2000a). Laccase reaction with 100 μg/ml PCP produced polymers of 80 000 MW with the formation of *ortho*- and *para*-chloranils.

Laccase from *Pyricularia oryzae* catalyzed an oxidative coupling reaction between 3-methyl-2-benzothiazolinone hydrazone (MBTH) and *ortho*-, *meta*-, and *para*-methoxyphenols and produced red azo compounds (Setti et al., 1999). The first step in the mechanism is the initiation of MBTH activation that undergoes an electrophilic substitution with *meta*-methoxyphenol in solution, enzymatic activated guaiacol, and chelated *para*-methoxyphenol at the catalytic site of the laccase. Different hydroxy- and methoxy-substituted phenols were oxidized by two laccase isozymes (laccase I and laccase II) from *Pleurotus eryngii* (Munoz et al., 1997). Both isozymes exhibited similar substrate specificities. Laccase I catalyzed higher rates of reactions than laccase II. Reactions of hydroquinones with laccase isozymes produce semiquinones, which in turn are partially converted to quinones via autooxidation. Two laccase isozymes (POXA1 and POXA2) produced by *Pleurotus ostreatus* have been purified and characterized (Palmieri et al., 1997). Both isozymes showed oxidation of syringaldazine and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) together with a variety of substituted phenols with concomitant reduction of oxygen, but POXA1 does not oxidize guaiacol. POXA2 exhibits the presence of four copper atoms per mole, while two zinc, one copper, and one iron atoms per mole are found in POXA1. POXA1 is unique in the neutral pI and anomalous metal content. Lack of absorbance at 600 nm classifies POXA1 as a novel "white" laccase.

The oxidative transformation of 2,6-dimethoxy-4-allyl phenol (DMAP) to the corresponding quinone methide takes place by mushroom tyrosinase (Krol and Bolton, 1997). Sugumaran and Bolton (1998) reported that laccase, not tyrosinase, is responsible for the formation of quinone methide from DMAP. The reaction involves an unusual 1,6-oxidation rather than the conventional 1,4-oxidation. Laccase is documented to oxidize syringaldazine, a structural analog of DMAP to quinone methide. A mechanism for the oxidation of DMAP by laccase has been proposed.

7.11.3.1 Influence of Cosubstrates. Industrial wastewaters are always heterogeneous, and the presence of highly reactive substrates can be beneficial for the detoxification of toxic compounds. Substrate removal can be enhanced due to a secondary chemical reaction between less reactive substrates and products formed during enzymatic oxidation of easily removed substrates. The aspects of the secondary chemical reaction have not been given good attention by researchers.

The reactive cosubstrates guaiacol and 2,6-dimethoxyphenol can influence the removal of chlorinated phenols by a laccase from *Trametes versicolor* (Roper et al., 1995). Removal of 2,4,5-TCP was enhanced from 8% to 85% by the addition of 20 mM guaiacol with *T. versicolor* laccase. Guaiacol also

improved the removal of 2,4-DCP from 38% to 70% and of 4-chlorophenol from 5% to 20%. Laccase of *Pleurotus eryngii* degraded 95% of 2,4-DCP after 6 hours in the presence of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT) (Rodriguez et al., 2004). However, lower efficiency of 60% degradation occurs in the absence of mediators.

Partially purified laccase of *Coriolus versicolor* shows transformation of chlorophenols in the order 2,4,6-TCP > 2,6-DCP > 2,4-DCP > 2-CP > 2,3-DCP (Itoh et al., 2000). Phenols with chlorine atoms at the *ortho-* and *para-*positions are more susceptible to laccase, and the reactivity increases with an increase in chlorine atoms. However, phenolic acids such as sinapinic acid increase degradation of 2,4,5-TCP, 2,4,6-TCP, 2,4-DCP, and 4-CP, whereas ferulic acid and *para*-coumaric acids inhibit degradation of 2,4,6-TCP, 2,3-, 2,4- and 2,6-DCPs, and 2-CP. The three phenolic acids have similar chemical structures, but their effects on the degradation of chlorophenols are different. Reaction schemes estimated for laccase-mediated transformation of 2,4-DCP with sinapinic and ferulic acid have been proposed. Two products were formed initially due to oxidation of ferulic acid by laccase (Carunchio et al., 2001).

A range of solid substrates for laccase production by *C. versicolor* has been evaluated for the development of a system for use in the bioremediation of chlorophenols in aqueous effluents (Ullah et al., 2000b). The substrates were wood chips, cereal grains, and wheat husk and bran. Wheat husk and bran supported the highest amount of laccase, <25 U/g after 30 days of colonization. Laccase extracted from wheat husk and bran cultures removed 100% of 2,4-dichlorophenol (50 ppm) within 5 hours and 75 to 80% of PCP (50 ppm) within 24 hours. Pellets were formulated of wheat bran with biscuit flour to form a compact substrate for fungal immobilization.

7.11.3.2 Laccase Bioreactors. Soluble and immobilized laccase from *Rhizoctonia praticola* on Celite are used to precipitate phenols from solution (Shuttleworth and Bollag, 1986). The amount of substrate removed depends on both the substituent group and the position of substitution. Transformation of 2,4-dichlorophenol occurs by fungal laccase immobilized on soil and clay, and similar efficiencies in immobilized and free enzymes have been found (Ruggeiro et al., 1989). Laccase can be immobilized by entrapment in calcium–alginate gel beads and by covalent coupling to activated carbon (Davis and Burns, 1990, 1992). Leonowicz et al. (1988) demonstrated the useful pH range and increased resistance to extremes of temperature for laccase immobilized on glass beads. Naturally occurring and xenobiotic aromatic compounds were detoxified from aqueous suspensions by organic gel-entrapped tyrosinase, and laccase and enzymes were reused several times without a reduction in efficiency (Crecchio et al., 1995). Immobilized enzymes have wide applications in membrane bioreactors. Laccase bioreactors are also known to remove phenolics and chlorophenols (Table 7.8).

7.11.4 Miscellaneous Enzymes

Table 7.9 lists miscellaneous fungal enzymes and mechanisms for the degradation of various phenolic compounds. Nitration of phenols with HRP, lipoxidase, lactoperoxidase, chloroperoxidase (CPO), and *Coprinus cinereus* peroxidase (CIP) has been demonstrated (Budde et al., 2001). CPO has been found to be a poor nitration catalyst, lactoperoxidase to be less effective than other peroxidases, and peroxidase from *C. cinereus* to be as effective as lipoxidase from soybean. CPO from *Caldariomyces fumago* catalyzed the chlorination of different phenolic compounds (Wannstedt et al., 1990). The metabolic products of several reactions and the percentages have been measured. Phenols have been polymerized and precipitated in aqueous solutions after treatment with CIP and H_2O_2 (Kauffmann et al., 1999). Expression of recombinant CIP in *Aspergillus oryzae* (Petersen et al., 1993), comparison of wild-type, recombinant, and three mutants of CIP (Veitch et al., 1994), and detailed comparison of wild-type CIP and its Asp245-Asn (D245N) mutant (Smulevich et al., 1996; Veitch et al., 1996), have shown the importance of CIP for phenol removal in industrial wastewaters. Gomez-Toribio et al. (2001) investigated the production of H_2O_2 during oxidation of three lignin-derived hydroquinones by the ligninolytic versatile peroxidase (VP) by *Pleurotus eryngii*. A wide range of phenols, including hydroquinones, can be oxidized by VP, either directly similar to horseradish peroxidase (HRP), or indirectly [Mn(II) to Mn(III) oxidation] similar to MnP. *P. eryngii* VP in the presence of Mn(II) degrades 65% of 50 μM 2,4-DCP within 1 hour (Rodriguez et al., 2004). However, 100% degradation of 2,4-DCP occurred after 2 hours by VP in the absence of Mn(II).

Extracellular diphenol oxidases from *Pleurotus ostreatus* are produced in culture broths supplemented with solid straw or tobacco juice (Garzillo et al., 1992). Palmieri et al. (1993) characterized three different phenol oxidases produced by *P. ostreatus*. This knowledge can be used in the development of bioreactor for wastewater treatment. Bonomo et al. (1998) isolated two isoforms of laccase as the predominant phenol oxidases by the white-rot fungus *Rigidoporus lignosus*. A specific phenol oxidase, *N*-acetyl-6-hydroxytryptophan oxidase, produced during conidiophore development in *Aspergillus nidulans* has been purified (Birse and Clutterbuck, 1990). A temperature-sensitive mutant produces a thermolabile enzyme that can oxidize *para*-cresol.

In recent years, several plant and animal enzymes have been used in the treatment of phenols. Ricoux et al. (2001) showed the ability of microperoxidase 8 (MP8) to catalyze the nitration of phenol by nitrite. Soybean peroxidase (SBP) catalyzes the precipitation and polymerization of aqueous phenols, and this treatment is less expensive than HRP.

TABLE 7.9 Fungal Enzymes for Degradation of Phenols

Fungus	Enzyme	Substrate(s)	Mechanism	Reference
Caldariomyces fumago	Chloroperoxidase	Phenols	Biohalogenation	Wannstedt et al., 1990
Coprinus cinereus	Peroxidase	Phenols	Nitration	Budde et al., 2001
C. cinereus	Peroxidase and H_2O_2	Phenols	Polymerization and precipitation	Kauffmann et al., 1999
Pleurotus eryngii	Versatile peroxidase	Phenols and hydroquinones	Oxidation	Gomez-Toribio et al., 2001
P. eryngii	Versatile peroxidase	2,4-DCP	Oxidation	Rodriguez et al., 2004
Bjerkandera adusta	Versatile peroxidase	PCP	Oxidation	Davila-Vazquez et al., 2005
P. eryngii	Aryl-alcohol oxidase	Benzyl, cinnamyl, naphthyl, and aliphatic unsaturated alcohols	Oxidation	Guillen et al., 1992
Phanerochaete chrysosporium	Methyltransferase	Phenols, benzoic acids, —OH substituents, etc.	Methylation	Jeffers et al., 1997
Penicillium simplicissimum	Aryl-alcohol oxidase	Vanillyl alcohol	Oxidation	de Jong et al., 1992
P. simplicissimum	Vanillyl-alcohol oxidase	Hydroxybenzyl alcohols, amines, glycols, allylphenols, etc.	Oxidation, deamination, hydride transfer	Fraaije et al., 1995
Aspergillus nidulans	N-Acetyl-6-hydroxytryptophan oxidase	Cresol	Oxidation	Birse and Clutterbuck, 1990

7.12 FUNGAL TRANSFORMATION OF PENTACHLOROPHENOL IN SOILS

Only during the last decade has fungal transformation of pentachlorophenol in soils received some consideration. However, more work is required to elucidate the process of transformation in soils. In general, the mycorrhizal fungi show less tolerance to creosote than the saprophytic fungi (Richter et al., 2003). Of saprophytic fungi, *Irpex lacteus*, *Neolentinus lepideus*, *Ouedemansiella radicata*, *P. chrysosporium*, *Postia placenta*, and *T. versicolor* exhibit the greatest tolerance to creosote. Mycorrhizal fungi such as *Cenococcum geophilum*, one isolate each of *Laccaria bicolor* and *L. laccata*, and *Suillus granulatus* show the highest tolerance to creosote. Rates of degradation or mineralization and metabolic products of PCP in soils by fungi are organized in Table 7.10.

7.12.1 Bound Residue Formation

Humic substances are amorphous, partly aromatic, and mostly dark-colored materials that are formed in soil by condensation of polyphenols and quinones. Microbial transformations such as decarboxylation and demethoxylation release different types of phenolic compounds, which are precursors of humic acid polymers. Covalent incorporation of chlorophenols into soil organic matter can occur via free radical–mediated copolymerization and/or its degradation product and organic precursors of humic and fulvic acids. A significantly lower amount of such incorporation occurs in *Pleurotus ostreatus* (Bogan et al., 1999). About 90% transformation of PCP to nonextractable residues is noted in some model soils. *P. ostreatus* has the ability to catalyze the incorporation of PCP into bound residues. Spiking of low concentration of PCP into uncontaminated soil is noted. The binding of PCP to humic materials seems to be catalyzed by oxidative enzymes. In vitro studies have shown the binding of humic acid and fulvic acid by peroxidases and laccases (Bollag, 1991; Bollag et al., 1992).

A large part (34 to 65%) of [^{14}C]PCP in contaminated soil is bound to soil organic matter by white-rot fungi (Ruttimann-Johnson and Lamar, 1997). The highest amounts (20 to 36%) of PCP bind with humic acid. Fungal cultures show lower binding to fulvic acid (8.7 to 17.5%) and humin (5 to 11%). *P. ostreatus* is the most efficient in binding (65%) to all three fractions of humic materials, followed by *Irpex lacteus*, *T. versicolor*, and *Bjerkandera adusta*. Mineralization of PCP in soils is low. Of three white-rot fungi, *I. lacteus* mineralizes the highest amounts, converting 8.8% of the PCP to CO_2 after 9 weeks. The mineralization rates are highest between 10 and 25 days. The rate of methylation of PCP to pentachloroanisole (PCA) is in the low range (0 to 6.8%).

TABLE 7.10 Fungal Transformation of Pentachlorophenol in Soils

Fungus	PCP Conc.	Degradation/ Mineralization Rate (%)	Metabolic Products (%)	Enzymatic Activities	Duration (weeks/ days/hours)	Reference
Trametes versicolor *Irpex lacteus* *Bjerkandera adusta*	[^{14}C]PCP, 75 µg/g	7.2 as $^{14}CO_2$ 8.8 as $^{14}CO_2$ 3.3 as $^{14}CO_2$	Pentachloroanisole (6.8) Pentachloroanisole (2.5) Pentachloroanisole (4.4)		9 w	Ruttimann-Johnson and Lamar, 1997
Lentinula edodes (monocultures)	PCP, 122.35 mg/kg	99	Pentachloroanisole, tetrachloroanisole, and tetrachlorophenol	Phenol oxidase, 195.5 U/g and MnP, 138.4 U/g	10 w	Okeke et al., 1997
L. edodes (mixed culture)	PCP, 122.35 mg/kg	42	Pentachloroanisole, tetrachloroanisole, and tetrachlorophenol	Phenol oxidase, 70.3 U/g and MnP, 80 U/g		
Phanerochaete chrysosporium or *P. sordida*	PCP, 250–400 µg/g strongly alkaline soil	88–91	Pentachloroanisole		6.5 w	Lamar and Glaser, 1990
Trametes hirsuta *P. chrysosporium*	PCP, 672 µg/g strongly acidic soil	89 67	Pentachloroanisole Pentachloroanisole		8 w	
Rhizopus nigricans *Gloeophyllum striatum* *Gloeophyllum trabeum* *T. versicolor* *T. versicolor*	PCP, 12.5 mg/l PCP, 10 µM PCP, 10 µM PCP, 10 µM PCP, 30 ppm	60 27 as $^{14}CO_2$ 19 of $^{14}CO_2$ 43 of $^{14}CO_2$ 1.25 as CO_2		Laccase, MnP, MIP MnP 243 and laccase, 591 U/nM substrate oxidized per minute per gram of soil	24 h 6 w 4 d	Cortes et al., 2002 Fahr et al., 1999 Boyle, 1995
T. versicolor	Aged contaminated soil PCP, 800 mg/kg	88			52 w	Walter et al., 2005

7.12.2   Degradation and Mineralization

PCP transformation results in the formation of toxic breakdown products in soils inoculated with *Phanerochaete chrysosporium* (McGrath and Singleton, 2000). PCP concentrations in all nonsterile soils are reduced from 250 mg/kg to 2 mg/kg after 6 weeks of incubation at 25°C. No PCP transformation is found in sterile soils. The dehydrogenase activity decreases considerably in all soils containing PCP and does not recover after 6 weeks, despite the decrease in PCP levels. Soil methanol extracts from PCP-contaminated soils inoculated with the fungus prove completely inhibitory for the growth of *Bacillus megaterium* after 6 weeks. PCP is also transformed in sterilized and nonsterilized soils inoculated with solid substrate cultures of *Lentinus* (*Lentinula*) *edodes* LE2 (Okeke et al., 1997). The kinetics of biotransformation by monocultures demonstrates rapid PCP depletion within the initial 4 weeks, and 99% depletion in 10 weeks. Phenol oxidase and MnP activities rise rapidly, reaching 195.5 U/g and 138.4 U/g after 2 weeks, respectively. The disappearance of PCP and biomass (ergosterol) accumulation are strongly correlated. In mixed cultures of nonsterilized soils, 42% PCP disappeared and enzyme activities were markedly lower after 10 days. High-resolution gas chromatography and mass spectrometry (HRGC/MS) reveals the presence of PCP transformation products such as PCA, tetrachloroanisole, and tetrachlorophenol in the first 4 weeks in both sterilized and nonsterilized soils. *L. edodes* monoculture shows higher dechlorination efficiency (29.50%) than does a mixed culture (22.40%).

An overall 88 to 91% PCP was depleted from the upper 25 cm of sandy gravel contaminated soil of pH 9.6 inoculated with *P. chrysosporium* or *Phanerochaete sordida* in 6.5 weeks (Lamar and Glaser, 1990). Fungal inocula are grown completely on wood chips. This involves mineralization, methylation to PCA, and formation of soil-bound residues. Only 9 to 14% of the PCP is converted to PCA, and methylation is not the major route of PCP transformation. Inocula of each of three white-rot fungi (*P. chrysosporium*, *P. sordida*, and *Trametes hirsuta*) are also prepared from pure cultures grown on nutrient-fortified sawdust grain mixture. This treatment with *P. sordida* removes 89% of PCP in strongly acidic contaminated clay oil of pH 3.8 after 8 weeks.

Solid-state culture (SSC) can be employed in different bioremediation strategies in contaminated soils. Efficient bioremediation depends on an increase in the biomass of degrading fungi in the contaminated soils. Filamentous fungi can also be used in these processes. One advantage is that these fungi grow faster than Basidiomycetes, and often at low temperatures. *Rhizopus nigricans* isolated from paper mill effluent showed a high tolerance to grow in the presence of 100 mg PCP/l (Cortes et al., 2002). The fungus displayed a higher efficiency in the removal of PCP in SSC than in submerged culture. In SSC, 60% PCP degraded within 24 hours at an initial concentration of 12.5 mg PCP/l. The same strain degraded 66% of PCP in 144 hours at

the same initial concentration in submerged culture (Cortes et al., 2001). The maximum CO_2 release rate is 3.5 ml/h per gram idm at 0, 12.5, and 25 mg PCP/l. These features indicate that it is an interesting alternative for bioremediation processes. Moreover, *R. nigricans* degrades PCP with enzymes (phenol oxidases) that are more stable than the enzymatic systems (peroxidases) found in *P. chrysosporium* and other Basidiomycetes. Degradation of 2,4-DCP and PCP occurs on wheat straw cultures of the brown-rot fungi *Gloeophyllum striatum* and *G. trabeum* and the white-rot fungus *T. versicolor* (Fahr et al., 1999). *T. versicolor* reveals the highest mineralization (43%) of PCP after 6 weeks. Ligninolytic enzymes are not detected in *Gloeophyllum* on wheat straw, suggesting new evidence of a degradation mechanism independent of such enzymes. Wetzstein et al. (1997) also discussed the absence of ligninolytic enzymes in *Gloeophyllum striatum*. D'Souza et al. (1996) reported on the scarce evidence for the occurrence of ligninolytic enzymes in brown-rot fungi. However, *T. versicolor* produces MnP, MIP and laccase on wheat straw throughout the cultivation period with maximum activities after 1 week.

7.12.3 Fungal Augmentation

Fungal augmentation of high-quality inoculums with high potential can be introduced to contaminated soils. Preliminary methods have been developed for the production of fungal inocula for bioaugmentation of contaminated soils. These inocula can be in the form of pelleted solid substrates coated with fungal mycelium, prepared from cheap agricultural and industrial by-products. Pelleted inocula have certain advantages over fungal carriers used earlier such as corncobs, wood chips, or straw. Fungal inocula can be optimized in substrate composition to enhance fungal growth, degradation abilities, and competitiveness against native soil microbes. To optimize fungal growth and degradation ability, methods to measure biomass are yet to be developed. The difficulty of separating fungal mycelia from solid substrates leads to indirect methods for the estimation of biomass and activity. These include methods measuring cell constituents, biological activity, and nutrient consumption (Desgranges et al., 1991). The fluorescein diacetate (FDA) hydrolyzing activity assay is also employed for microbial biomass growth, which measures the activity of a number of enzymes.

The FDA assay is evaluated as an indicator of biological potential of solid pellet inoculums for bioaugmentation of contaminated soils with white-rot fungi (Lestan et al., 1996). The FDA activity of *Phanerochaete chrysosporium* grown in pellets correlates with mycelia dry weight and ergosterol content of fungal mycelium. In addition to optimum substrate composition, optimum temperature and pellet structure influence high metabolic activity, proliferation, and PCP tolerance of *P. chrysosporium*. *P. chrysosporium*, and *T. versicolor* introduced on PCP-contaminated soils on pellets with high biological potential and high nitrogen content (C:N ratio of 50:1) are unable to remove

PCP more efficiently than fungi introduced on pellets with a lower biological potential (C:N ratio of 309:1). However, PCP is significantly transformed to PCA using pellets of lower biological potential. *T. versicolor* inoculated in soil with pellets of high biological potential shows higher MnP activity than to soil inoculated with pellets of lower biological potential.

A practical method is developed to promote the growth of white-rot fungi in soil without the use of large quantities of amendments or inoculum (Boyle, 1995). In *T. versicolor*, MnP and laccase activities increase with addition of either a carbon or nitrogen source and are enhanced by the addition of both supplements. Alfalfa is a more effective source of these nutrients than other amendments. Fungi colonize alfalfa-amended nonsterile soil due to competition from other microorganisms. Mineralization of [^{14}C]PCP is more rapid in soil amended with alfalfa and benomyl and inoculated with *T. versicolor*. About 1.25% of the 30 ppm PCP in the soil degraded to CO_2 per day during the 4-day period. The maximum MnP and laccase activities were 243 and 591 U/nM substrate oxidized per minute per gram respectively, on the third day of incubation. Based on a different growth assay (epifluorescence microscopy image analysis), Morgan et al. (1993) concluded that an amendment of ground wheat straw (similar to alfalfa) is effective at stimulating growth of white-rot fungi in soils.

7.13 CYTOCHROME P450 SYSTEMS IN DEGRADATION OF PHENOLS

The involvement of cytochrome P450 to catalyze a wide range of monooxygenase reactions, including endogenous and xenobiotic substrates, is well established. The metabolism of 2,4-DCP has been examined using microsomal fractions and whole cells of *Saccharomyces cerevisiae* strain AH22 containing human cytochrome P450 3A4 (Mehmood et al., 1997). Type I substrate binding spectrum with a K_s value of 75 μM is noted with 2,4-DCP. 2,4-DCP is metabolized efficiently into two metabolites: 2-chloro-1,4-hydroxyquinone and 2-chloro-1,4-benzoquinone in microsomal fractions and whole cells of yeast expressing human cytochrome P450 3A4. Biotransformation by whole cells also leads to the formation of a further metabolite, 1,2,4-hydroxybenzene. The metabolism depends on nicotinamide adenine dinucleotide phosphate (NADPH) in microsomal fractions, and no activity is detected in control microsomal fractions or whole cells of control transformants. Based on the identification of metabolites, a pathway of 2,4-DCP biotransformation by yeast expressing human P450 3A4 is proposed. The first step in this pathway is the hydroxylation of 2,4-DCP to metabolite, 2-chloro-1,4-hydroxyquinone, and this reaction has also been reported in white-rot fungi (Valli and Gold, 1991). The other two metabolites are 2-chloro-1,4-benzoquinone, anticipated as a product of peroxidase metabolism of 2,4-DCP, and 1,2,4-hydroxybenzene as P450 3A4-mediated. It is possible that yeast

microsomes contain dehydrogenase acting on 2-chloro-1,4-hydroxyquinone. Either or both chlorine atoms from 2,4-DCP can also be removed by P450 3A4.

Cytochrome P450s of *Phanerochaete chrysosporium* are involved in 23 hydroxylation and two deethylation reactions with 11 substrates, indicating unique and diverse functions (Matsuzaki and Wariishi, 2004). At least 148 P450 genes exist in *P. chrysosporium* genome (Martinez et al., 2004), and functional diversity is expected.

7.14   CONCLUSIONS AND FUTURE PERSPECTIVES

A number of fungi have been screened, isolated, and the mechanism of action and pathways of metabolism of various phenols studied and elucidated. Enzymes involved in these pathways have also been isolated; however, little is known about the genes encoding these enzymes.

Different types of fungal bioreactors are established for the treatment of phenolic wastewaters. Biofilms of white-rot fungi can be employed that show less susceptibility to variation in the chemical composition of wastewater feeds. Considerable success has been achieved in the immobilization of fungal cells in sorbent (Godjevargova et al., 1998) and membrane bioreactors (Luke and Burton, 2001). The effectiveness of fungal laccases immobilized in membrane reactors (Lante et al., 2000) has been demonstrated to couple both the effects of enzyme action and those due to membrane filtration. Limited knowledge suggests enhanced removal of chlorophenols in the presence of cosubstrates by laccase and can open new horizons for the rapid treatment of heterogeneous wastewaters. However, the optimization of culture conditions, feed composition, and operation needs to be fully investigated before its use in bioreactors. In fact, the system should be designed in a way that can be beneficial for the long-term operation of bioreactors.

The potential of certain fungi in field bioremediation can be enhanced by a number of factors. This includes an increase in the inoculum biomass/soil ratio and supplementation of the soil culture system, such as bark chips, straw, or sawdust. Partial sterilization of soil through fumigation may also be useful for the process. PCP is depleted in both the presence and absence of natural soil microflora by *Lentinula edodes* biomass in sawdust (Okeke et al., 1997). Engineered soil cells lead to successful mycoremediation of aged PCP-contaminated soil by *Trametes versicolor* (Walter et al., 2005). Certain soil fungi of Deuteromycetes can also be involved in the self-purification processes in soil. These soil fungi metabolize halophenols to form a less toxic dienelactone or polymerize after oxidation, resulting in the formation of humus-like insoluble compounds. Moreover, it is useful to screen efficient or sensitive strains that can be employed in soil remediation.

Advancement in molecular biotechnology has not thrown any light on the recombinant fungi related to biodegradation of phenols and related com-

pounds. Fungal genes specific for the degradation of phenols have not been isolated. However, three purified proteins, PcpA, PcpB, and PcpC, are isolated in *Flavobacterium* sp. ATCC 39723, which are involved in the breakdown of PCP and other halogenated phenols (Orser et al., 1997). Three cloned genes, pcpA, pcpB, and pcpC, encoding these proteins and two additional genes, pcpD and pcpR, involved in PCP degradation have been reported. A great future lies in the isolation of such proteins and genes in fungi that can be used in mycoremediation applications for the detoxification of different types of phenols.

REFERENCES

Abelskow, A.K., A.T. Smith, C.B. Rasmussen, H.B. Dunford, and K.G. Welinder (1997) pH dependence and structural interpretation of the reactions of *Coprinus cinereus* peroxidase with hydrogen peroxide, ferulic acid, and 2,2′-azinobis. *Biochemistry* **36**: 9453–9463.

Aiken, B.S., and B.E. Logan (1996) Degradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium* grown in ammonium lignosulphonate medium. *Biodegradation* **7**: 175–182.

Akta, N., H. Cicek, A.T. Unal, G. Kibarer, N. Kolankaya, and A. Tanyolac (2001) Reaction kinetics for laccase-catalyzed polymerization of 1-naphthol. *Bioresour. Technol.* **80**: 29–36.

Al-Kassim, L., K.E. Taylor, J.K. Bewtra, and N. Biswas (1994a) Optimization of phenol removal by a fungal peroxidase from *Coprinus macrorhizus* using batch, continuous and discontinuous semibatch reactors. *Enzyme Microb. Technol.* **16**: 120–124.

Al-Kassim, L., K.E. Taylor, J.A. Nicell, J.K. Bewtra, and N. Biswas (1994b) Enzymatic removal of selected aromatic contaminants from wastewater by a fungal peroxidase from *Coprinus macrorhizus* using batch reactors. *J. Chem. Technol. Biotechnol.* **61**: 179–182.

Alleman, B.C., B.E. Logan, and R.L. Gilbertson (1995) Degradation of pentachlorophenol by fixed films of white rot fungi in rotating tube bioreactors. *Water Res.* **29**: 61–67.

Alnaizy, R., and A. Akgerman (2000) Advanced oxidation of phenolic compounds. *Adv. Environ. Res.* **4**: 233–244.

Arana, J., E.T. Rendon, J.M.D. Rodriguez, J.A.H. Melian, O.G. Diaz, and J.P. Perez (2001) Highly concentrated phenolic wastewater treatment by the photo-Fenton reaction, mechanism study by FTIR-ATR. *Chemosphere* **44**: 1017–1023.

Banerjee, G. (1997) Treatment of phenolic wastewater in RBC reactor. *Water Res.* **31**: 705–714.

Bastos, A.E.R., D.H. Moon, G.R. Furlan, V.L. Tornisiello, and S.M. Tsai (1996) Methodology for the selection and isolation of phenol degrading microorganisms. *Int. Biodeterior. Biodegrad.* **37**: 123.

Bastos, A.E.R., G.R. Furlan, V.L. Tornisiello, S.M. Tsai, and D.H. Moon (1997) A comparison of two radiometric methods to investigate the biodegradation of phenol by *Candida nitrativorans*. *J. Microbiol. Methods* **29**: 177–183.

Bier, F.F., E. Ehrentreich-Forster, F.W. Scheller, A. Makower, et al. (1996a) Ultrasensitive biosensors. *Sens. Actuators B* **33**: 5–12.

Bier, F.F., E. Ehrentreich-Forster, A. Makower, and F.W. Scheller (1996b) An enzymatic amplification cycle for high sensitive immunoassay. *Anal. Chim. Acta* **328**: 27–32.

Birse, C.E., and A.J. Clutterbuck (1990) *N*-Acetyl-6-hydroxytryptophan oxidase, a developmentally controlled phenol oxidase from *Aspergillus nidulans*. *J. Gen. Microbiol.* **136**: 1725–1730.

Bogan, B.W., R.T. Lamar, W.D. Burgos, and M. Tien (1999) Extent of humification of anthracene, fluoranthene, and benzo[*a*]pyrene by *Pleurotus ostreatus* during growth in PAH-contaminated soils. *Lett. Appl. Microbiol.* **28**: 250–254.

Bokern, M., and H. Harms (1997) Toxicity and metabolism of 4-nonylphenol in different plant systems. *Environ. Sci. Technol.* **31**: 1849–1854.

Bollag, J.-M. (1991) Enzymatic binding of pesticide degradation products to soil organic matter and their possible release: pesticide transformation products fate and significance in the environment. In: *American Chemical Society Symposia Series 459*, L. Somasundaram and J.R. Coats, eds. American Chemical Society, Washington, DC, pp. 122–132.

Bollag, J.-M., C.J. Myers, and R.D. Minard (1992) Biological and chemical interactions of pesticides with soil organic matter. *Sci. Total Environ.* **123–124**: 205–217.

Bonomo, R.P., A.M. Boudet, R. Cozzolino, E. Rizzarelli, et al. (1998) A comparative study of two isoforms of laccase secreted by the "white-rot" fungus *Rigidoporus lignosus*, exhibiting significant structural and functional differences. *J. Inorg. Biochem.* **71**: 205–211.

Boominathan, K., and A. Mahadevan (1989) Dissimilation of aromatic substances by fungi. *Z. Mikrobiol.* **144**: 37–45.

Boyle, C.D. (1995) Development of a practical method for inducing white-rot fungi to grow into and degrade organopollutants in soil. *Can. J. Microbiol.* **41**: 345–353.

Budde, C.L., A. Beyer, I.Z. Munir, J.S. Dordick, and Y.L. Khmelnitsky (2001) Enzymatic nitration of phenols. *J. Mol. Catal. B* **15**: 55–64.

Burton, S.G., A. Boshoff, W. Edwards, and P.D. Rose (1998) Biotransformation of phenols using immobilized polyphenol oxidase. *J. Mol. Catal. B* **5**: 411–416.

Cabanes, J., F. Garcia-Canovas, J.A. Lozano, and F. Garcia-Carmona (1987) A kinetic study melanization pathway between L-tyrosinase and dopachrome. *Biochim. Biophys. Acta* **923**: 187–195.

Cain, R.B. (1980) The uptake and catabolism of lignin-related aromatic compounds and regulation in microorganisms. In: *Lignin Biodegradation: Microbiology, Chemistry and Potential Applications*, T.K. Kirk, T. Higuchi, and H. Chang, eds. CRC Press, Boca Raton, FL, Vol. 1, pp. 21–60.

Cain, R.B. (1987) Aromatic metabolism by mycelial organisms: actinomycete fungal strategies. In: *Microbial Metabolism and the Carbon Cycle*, S.R. Hagedorn, R.S. Hanson, and D.A. Kunz, eds. Horwood, London.

Campanella, L., G. Favero, and M. Tomassetti (1995) Immobilized yeast cells biosensor for total toxicity testing. *Sci. Total Environ.* **171**: 227–234.

Canofeni, S., S.S. Di, J. Mela, and R. Pilloton (1994) Comparison of immobilization procedures for development of an electrochemical PPO-based biosensor for online monitoring of a depuration process. *Anal. Lett.* **27**: 1659–1669.

Carneiro, A., A. Abreu, D.V. Evtuguin, C.P. Neto, G. Guebitz, and A.C. Paulo (2000) Polyoxometalates as promoters of laccase-assisted reactions. *J. Mol. Catal. B* **9**: 293–295.

Carunchio, F., C. Crescenzi, A.M. Girelli, A. Messina, and A.M. Tarola (2001) Oxidation of ferulic acid by laccase: identification of the products and inhibitory effects of some dipeptides. *Talanta* **55**: 189–200.

Chang, B.-V., W.-B. Wu, and S.-Y. Yuan (1997) Biodegradation of benzene, toluene, and other aromatic compounds by *Pseudomonas* sp. D8. *Chemosphere* **35**: 2807–2815.

Chattonet, P., D. Dubourdieu, J. Boidron, and M. Pons (1992) The origin of ethyl phenols in wines. *J. Sci. Food Agric.* **60**: 165–178.

ChemExpo (1999) chemexpo.com/news/PROFILE990329.cfm.

Chiu, S.W., M.L. Ching, K.L. Fong, and D. Moore (1998) Spent oyster mushroom substrate performs better than many mushroom mycelia in removing the biocide pentachlorophenol. *Mycol. Res.* **102**: 1553–1562.

Chun, H., W. Yizhong, and T. Hongxiao (2000) Destruction of phenol aqueous solution by photocatalysis or direct photolysis. *Chemosphere* **41**: 1205–1209.

Chung, N., and S.D. Aust (1995) Veratryl alcohol–mediated indirect oxidation of phenol by lignin peroxidase. *Arch. Biochem. Biophys.* **316**: 733–737.

Chung, Y., and K. Lee (2001) Separation and determination of eleven environmental protection agency priority phenols by reversed-phase capillary high-performance liquid chromatography with indirect fluorophotometric detection and solid phase extraction. *Microchem. J.* **69**: 45–54.

Claußen, M., and S. Schmidt (1998) Biodegradation of phenol and *p*-cresol by the hyphomycete *Scedosporium apiospermum. Res. Microbiol.* **149**: 399–406.

Clauβen, M., and S. Schmidt (1999) Biodegradation of phenylbenzoate and some of its derivatives by *Scedosporium apiospermum. Res. Microbiol.* **150**: 413–420.

Clement, P., V. Matus, L. Cardenas, and B. Gonzalez (1995) Degradation of trichlorophenols by *Alcaligenes eutrophus* JMP134. *FEMS Microbiol. Lett.* **127**: 51–55.

Cooksey, C.J., P.J. Garratt, E.J. Land, C.A. Ramsden, and P.A. Riley (1998) Tyrosinase kinetics: failure of the auto-activation mechanism of monohydric phenol oxidation by rapid formation of a quinomethane intermediate. *Biochem. J.* **333**: 685–691.

Cortes, D.V., R. Bernal, and A. Tomasini (2001) Efecto de las condiciónes de cultivo sumergido en la degradación de pentaclorofenol. *Inform. Tecnol.* **12**: 75–80.

Cortes, D.V., J. Barrios-Gonzalez, and A. Tomasini (2002) Pentachlorophenol tolerance and removal by *Rhizopus nigricans* in solid-state culture. *Process Biochem.* **37**: 881–884.

Corti, A., S. Frassinetti, G. Vallini, S.D. Antone, C. Fichi, and R. Solaro (1995) Biodegradation of nonionic surfactants. I. Biotransformation of 4-(1-nonyl)phenol by a *Candida maltosa* isolate. *Environ. Pollut.* **90**: 83–87.

Cosnier, S., and I.C. Popescu (1996) Poly(amphiphilic pyrrole)-tyrosinase-peroxidase electrode for amplified flow injection-amperometric detection of phenol. *Anal. Chim. Acta* **319**: 145–152.

Crecchio, C., P. Ruggiero, and M. Pizzigallo (1995) Polyphenoloxidases immobilized in organic gels: properties and applications in the detoxification of aromatic compounds. *Biotechnol. Bioeng.* **48**: 585–591.

Darby, J.M., D.G. Taylor, and D.J. Hopper (1987) Hydroquinone as the ring-fission substrate in the catabolism of 4-ethylphenol and 4-hydroxyacetophenone by *Pseudomonas putida* JD1. *J. Gen. Microbiol.* **133**: 2137–2146.

Davila-Vazquez, G., R. Tinoco, M.A. Pickard, and R. Vazquez-Duhalt (2005) Transformation of halogenated pesticides by versatile peroxidase from *Bjerkandera adusta*. *Enzyme Microb. Technol.* **36**: 223–231.

Davis, S., and R.G. Burns (1990) Decolorization of phenolic effluents by soluble and immobilized phenol oxidaes. *Appl. Microbiol. Biotechnol.* **32**: 721–726.

Davis, S., and R.G. Burns (1992) Covalent immobilization of laccase on activated carbon for phenolic effluent treatment. *Appl. Microbiol. Biotechnol.* **37**: 474–479.

Dec, J., and J.-M. Bollag (1990) Detoxification of substituted phenols by oxidoreductive enzymes through polymerization reactions. *Arch. Environ. Contam. Toxicol.* **19**: 543–550.

Dec, J., and J.-M. Bollag (1994) Dehalogenation of chlorinated phenols during oxidative coupling. *Environ. Sci. Technol.* **28**: 484–490.

de Jong, E., W.J.H. van Berkel, R.P. van der Zwan, and J.A.M. de Bont (1992) Purification and characterization of vanillyl-alcohol oxidase from *Penicillium simplicissimum*: a novel aromatic alcohol oxidase containing covalently bound FAD. *Eur. J. Biochem.* **208**: 651–657.

Deschler, C., R. Duran, M. Junqua, C. Landou, J.-C. Salvado, and P. Goulas (1998) Involvement of 3,4-dichlorophenol hydroxylase in degradation of 3,4-dichlorophenol by the white rot fungus *Phanerochaete chrysosporium*. *J. Mol. Catal. B* **5**: 423–428.

Desgranges, C., C. Vergoignan, M. Georges, and A. Durand (1991) Biomass estimation in solid state cultivation. I. Manual biochemical methods. *Appl. Microbiol. Biotechnol.* **35**: 200–205.

De Wulf, O., P. Thonart, P. Gaignage, M. Marlier, A. Paris, and M. Paquot (1986) Bioconversion of vanillin to vanillyl alcohol by *Saccharomyces cerevisiae*. In: *Proceedings of Biotechnology and Bioengineering Symposium 17*, May 13–16, Gatlinburg, TN., Wiley, New York, pp. 605–615.

Droste, R.L., K.J. Kennedy, J. Lu, and M. Lentz (1998) Removal of chlorinated phenols in upflow anaerobic sludge blanket reactors. *Water Sci. Technol.* **38**: 359–367.

D'Souza, T.M., K. Boominathan, and C.A. Reddy (1996) Isolation of laccase gene-specific sequences from white rot and brown rot fungi by PCR. *Appl. Environ. Microbiol.* **62**: 3739–3744.

Edlin, D.A.N., A. Narbad, R. Dickinson, and D. Lloyd (1995) The biotransformation of simple phenolic compounds by *Brettanomyces anomalus*. *FEMS Microbiol. Lett.* **125**: 311–316.

Edwards, W., R. Bownes, W.D. Leukes, E.P. Jacobs, and R. Saderson, et al. (1999a) A capillary membrane bioreactor using immobilized polyphenol oxidase for the removal of phenols from industrial effluents. *Enzyme Microb. Technol.* **24**: 209–217.

Edwards, W., W.D. Leukes, P.D. Rose, and S.G. Burton (1999b) Immobilization of polyphenol oxidase on chitosan-coated polysulphone capillary membranes for phenolic effluent bioremediation. *Enzyme Microb. Technol.* **25**: 769–773.

Ehlers, G.A., and P.D. Rose (2005) Immobilized white-rot fungal biodegradation of phenol and chlorinated phenol in trickling packed-bed reactors by employing sequencing batch operation. *Bioresour. Technol.* **96**: 1264–1275.

Ellis, M.B. (1971) *Dematiaceous Hyphomycetes.* CMI, Kew, UK.

Evangelista, R.A., H.L. Allen, and R.M. Manuel (1990) Treatment of phenol and cresol contaminated soil. *J. Hazard. Mater.* **25**: 343–360.

Fahr, K., H.-G. Wetzstein, R. Grey, and D. Schlosser (1999) Degradation of 2,4-dichlorophenol and pentachlorophenol by two brown rot fungi. *FEMS Microbiol. Lett.* **175**: 127–132.

Fenoll, L.G., J.N. Rodriguez-Lopez, F. Garcia-Sevilla, J. Tudela, et al. (2000) Oxidation by mushroom tyrosinase of monophenols generating slightly unstable *o*-quinones. *Eur. J. Biochem.* **267**: 5865–5878.

Ferro, A.M., R.C. Sims, and B. Bugbee (1994) Hycrest crested wheatgrass accelerates the degradation of pentachlorophenol in soil. *J. Environ. Qual.* **23**: 272–281.

Fialova, A., E. Boschke, and T. Bley (2004) Rapid monitoring of the biodegradation of phenol-like compounds by the yeast *Candida maltosa* using BOD measurements. *Int. Biodeterior. Biodegrad.* **54**: 69–76.

Fraaije, M.W., C. Veeger, and J.H. Van Berkel (1995) Substrate specificity of flavin-dependent vanillyl-alcohol oxidase from *Penicillium simplicissimum*: evidence for the production of 4-hydroxycinnamyl alcohols from 4-allylphenols. *Eur. J. Biochem.* **234**: 271–277.

Freire, R.S., N. Duran, and L.T. Kubota (2001) Effects of fungal laccase immobilization procedures for the development of a biosensor for phenol compounds. *Talanta* **54**: 681–686.

Fuhrmann, B., and U. Spohn (1998) An enzymatic amplification flow injection analysis (FIA) system for the sensitive determination of phenol. *Biosens. Bioelectron.* **13**: 895–902.

Fukuda, T., H. Uchida, Y. Takashima, T. Uwajima, et al. (2001) Degradation of bisphenol A by purified laccase from *Trametes villosa*. *Biochem. Biophys. Res. Commun.* **284**: 704–706.

Gaisford, W.C., N.J. Richardson, B.G.D. Haggett, and D.M. Rawson (1991) Microbial biosensors for environmental monitoring. *Biochem. Soc. Trans.* **19**: 15–18.

Garzillo, A.M., S.D. Paolo, G. Burla, and V. Buonocore (1992) Differently-induced extracellular phenol oxidases from *Pleurotus ostreatus*. *Phytochemistry* **31**: 3685–3690.

Gerlach, R.W., and J.M. Van Emon (1997) Site evaluation of field portable pentachlorophenol immunoassay. *Chemosphere* **35**: 2727–2749.

Ghindilis, A.L., V.P. Gavrilova, and A.I. Yaropolov (1992) Laccase-based biosensor for determination of polyphenols: determination of catechols in tea. *Biosens. Bioelectron.* **7**: 127–131.

Godjevargova, T., Z. Aleksieva, D. Ivanova, and N. Shivarova (1998) Biodegradation of phenol by *Trichosporon cutaneum* cells covalently bound to polyamide granules. *Process Biochem.* **33**: 831–835.

Golovleva, L.A., O. Zaborina, R. Pertsova, B. Baskunov, Y. Schurukhin, and S. Kuzmin (1991–1992) Degradation of polychlorinated phenols by *Streptomyces rochei* 303. *Biodegradation* **2**: 201–208.

Gomez-Toribio, V., A.T. Martinez, M.J. Martinez, and F. Guillen (2001) Oxidation of hydroquinones by the versatile ligninolytic peroxidase from *Pleurotus eryngii*: H_2O_2 generation and the influence of Mn^{2+}. *Eur. J. Biochem./FEBS* **268**: 4787–4793.

Gonzalez, G., G. Herrera, M.T. Garcia, and M. Pena (2001) Biodegradation of phenolic industrial wastewater in a fluidized bed bioreactor with immobilized cells of *Pseudomonas putida*. *Bioresour. Technol.* **80**: 137–142.

Grabski, A.C., H.J. Grimek, and R.R. Burgess (1998) Immobilization of manganese peroxidase from *Lentinula edodes* and its biocatalytic generation of Mn^{III}-chelate as a chemical oxidant of chlorophenols. *Biotechnol. Bioeng.* **60**: 204–215.

Grey, R., C. Hofer, and D. Schlosser (1998) Degradation of 2-chlorophenol and formation of 2-chloro-1,4-benzoquinone by mycelia and cell-free crude culture liquids of *Trametes versicolor* in relation to extracellular laccase activity. *J. Basic Microbiol.* **38**: 371–382.

Guillen, F., A.T. Martinez, and M.J. Martinez (1992) Substrate specificity and properties of the aryl-alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii*. *Eur. J. Biochem./FEBS* **209**: 603–611.

Guiraud, P., R. Steiman, L. Ait-Laydi, and F. Seigle-Murandi (1999) Degradation of phenolic and chloroaromatic compounds by *Coprinus* spp. *Chemosphere* **38**: 2775–2789.

Gukasyan, G.S. (2002) Study of the kinetics of monophenols by tyrosinase: the effect of reducers. *Biochem. Biokhim.* **67**: 277–280.

Hammel, K.E., and P.J. Tardone (1988) The oxidative 4-dechlorination of polychlorinated phenols is catalyzed by extracellular fungal lignin peroxidases. *Biochemistry* **27**: 6563–6568.

Hamoudi, S., A. Sayari, K. Belkacemi, L. Bonneviot, and F. Larachi (2000) Catalytic wet oxidation of phenol over $Pt_xAg_{1-x}MnO_2/CeO_2$ catalysts. *Catal. Today* **62**: 379–388.

Hanne, L.F., L.L. Kirk, S.M. Appel, A.D. Narayan, and K.K. Bains (1993) Degradation and induction specificity in actinomycetes that degrade *p*-nitrophenol. *Appl. Environ. Microbiol.* **59**: 3505–3508.

Hebatpuria, V.M., H.A. Arafat, H.S. Rho, P.L. Bishop, N.G. Pinto, and R.C. Buchanan (1999) Immobilization of phenol in cement-based solidified/stabilized hazardous waste using regenerated activated carbon: leaching studies. *J. Hazard. Mater.* **70**: 117–138.

Heresztyn, T. (1986) Metabolism of volatile phenolic compounds from hydroxycinnamic acid by *Brettanomyces* yeast. *Arch. Microbiol.* **146**: 96–98.

Hill, G.A., B.J. Milne, and P.A. Nawrocki (1996) Cometabolic degradation of 4-chlorophenol by *Alcaligenes eutrophus*. *Appl. Microbiol. Biotechnol.* **46**: 163–168.

Hirano, T., Y. Honda, T. Watanabe, and M. Kuwahara (2000) Degradation of bisphenol A by the lignin-degrading enzyme, manganese peroxidase, produced by the white-rot basidiomycete *Pleurotus ostreatus. Biosci. Biotechnol. Biochem.* **64**: 1958–1962.

Hofmann, K.H., and F. Schauer (1988) Utilization of phenol by hydrocarbon assimilating yeasts. *Antonie Leeuwenhoek* **54**: 179–188.

Hofrichter, M., Friedemann, and W. Fritsche (1994) Unspecific degradation of halogenated phenols by the soil fungus *Penicillium frequentans* Bi 7/2. *J. Basic Microbiol.* **34**: 163–172.

Hublik, G., and F. Schinner (2000) Characterization and immobilization of the laccase from *Pleurotus ostreatus* and its use for the continuous elimination of phenolic pollutants. *Enzyme Microb. Technol.* **27**: 330–336.

Itoh, K., M. Fujita, K. Kumano, K. Suyama, and H. Yamamoto (2000) Phenolic acids affect transformations of chlorophenols by a *Coriolus versicolor* laccase. *Soil Biol. Biochem.* **32**: 85–91.

Ivanova, N., and L. Yotova (1993) Biotransformation of furfural by yeast cells covalently bound to cellulose granules. *Acta Biotechnol.* **13**: 79–82.

Jeffers, M.R., W.C. McRoberts, and D.B. Harper (1997) Identification of a phenolic 3-*O*-methyltransferase in the lignin-degrading fungus *Phanerochaete chrysosporium. Microbiology* **163**: 1975–1981.

Jensen, J. (1996) Chlorophenols in the terrestrial environment. *Rev. Environ. Contam. Toxicol.* **146**: 25–51.

Jones, K.H., P.W. Trudgill, and D.J. Hopper (1993) Metabolism of *p*-cresol by the fungus *Aspergillus fumigatus. Appl. Environ. Microbiol.* **59**: 1125–1130.

Jones, K.H., P.W. Trudgill, and D.J. Hopper (1994) 4-Ethylphenol metabolism by *Aspergillus fumigatus. Appl. Environ. Microbiol.* **60**: 1978–1983.

Jones, K.H., P.W. Trudgill, and D.J. Hopper (1995) Evidence of two pathways for the metabolism of phenol by *Aspergillus fumigatus. Arch. Microbiol.* **163**: 176–181.

Joshi, D.K., and M.H. Gold (1993) Degradation of 2,4,5-trichlorophenol by the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **59**: 1779–1785.

Jung, J., B. Sanji, S. Godbole, and S. Sofer (1993) Biodegradation of phenol: a comparative study with and without applying magnetic fields. *J. Chem. Technol. Biotechnol.* (*Oxford*) **36**: 73–76.

Kadhim, H., C. Graham, P. Barratt, C.S. Evans, and R.A. Rastall (1999) Removal of phenolic compounds in water using *Coriolus versicolor* grown on wheat bran. *Enzyme Microb. Technol.* **24**: 303–307.

Karam, J., and J.A. Nicell (1997) Potential applications of enzymes in waste treatment. *J. Chem. Tech. Biotechnol.* **69**: 141–153.

Katayama-Hirayama, K., S. Tobita, and K. Hirayama (1994) Biodegradation of phenol and monochlorophenols by yeast *Rhodotorula glutinis. Water Sci. Technol.* **30**: 59–66.

Kauffmann, C., B.R. Petersen, and M.J. Bjerrum (1999) Enzymatic removal of phenols from aqueous solutions by *Coprinus cinereus* peroxidase and hydrogen peroxide. *J. Biotechnol.* **73**: 71–74.

Keith, L.H., and W.A. Telliard (1979) Priority pollutants. I. A perspective view. *Environ. Sci. Technol.* **13**: 416–423.

Kennes, C., and J.M. Lema (1994) Simultaneous biodegradation of *p*-cresol and phenol by the basidiomycete *Phanerochaete chrysosporium*. *J. Ind. Microbiol.* **13**: 311–314.

Kim, M.H., and O.J. Hao (1999) Cometabolic degradation of chlorophenols by *Acinetobacter* species. *Water Res.* **33**: 562–574.

Kjalke, M., M.B. Andersen, P. Schneider, and B. Christensen, et al. (1992) Comparison of structure and activities of peroxidases from *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus*. *Biochim. Biophys. Acta* **1120**: 248–256.

Kjellen, K.G., and H.Y. Neujahr (1979) Immobilization of phenol hydroxylase. *Biotechnol. Bioeng.* **21**: 715–719.

Kjellen, K.G., and H.Y. Neujahr (1980) Enzyme electrode for phenol. *Biotechnol. Bioeng.* **22**: 299–310.

Konishi, K., and Y. Inoue (1972) Detoxification mechanism of pentachlorophenol by the laccase of *Coriolus versicolor*. *Mokuzai Gakkaishi* **18**: 463–469.

Kremer, S., and H. Anke (1997) Fungi in bioremediation. In: *Fungal Biotechnology*, T. Anke, ed. Chapman & Hall, Weinheim, Germany, pp. 275–295.

Kremer, S., O. Sterner, and H. Anke (1992) Degradation of pentachlorophenol by *Mycena avenacea* TA 8480: identification of initial dechlorinated metabolites. *Z. Naturfors.* **47**: 561–566.

Krivobok, S., J.-L. Benoit-Guyod, F. Seigle-Murandi, R. Steiman, and G.-A. Thiault (1994) Diversity in phenol-metabolizing capacity of 809 strains of Micromycetes. *Microbiologica* **17**: 51–60.

Krol, E.S., and J.L. Bolton (1997) Oxidation of 4-alkylphenols and catechols by tyrosinase: *ortho*-substituents alter the mechanism of quinoid formation. *Chem.-Biol. Interact.* **104**: 11–27.

Kuramitz, H., Y. Nakata, M. Kawasaki, and S. Tanaka (2001) Electrochemical oxidation of biphenol A: application to the removal of bisphenol A using a carbon fiber electrode. *Chemosphere* **45**: 37–43.

Lacher, N.A., K.E. Garrison, R.S. Martin, and S.M. Lunte (2001) Microchip capillary electrophoresis/electrochemistry. *Electrophoresis* **22**: 2526–2536.

Laine, M.M., and K.S. Jorgensen (1997) Effective and safe composting of chlorophenol-contaminated soil in pilot scale. *Environ. Sci. Technol.* **31**: 371–378.

Lamar, R.T., and J.A. Glaser (1990) Field evaluations of the remediation of soils contaminated with wood-preserving chemicals using lignin-degrading fungi. In: *Bioremediation of Chlorinated and Polycyclic Aromatic Compounds*, R.E. Hinchee, A. Leeson, L. Semprini, and S.S. Ong, eds. Lewis Publishers, Boca Raton, FL, pp. 239–247.

Lante, A., A. Crapisi, A. Krastanov, and P. Spettoli (2000) Biodegradation of phenols by laccase immobilized in a membrane reactor. *Process Biochem.* **36**: 51–58.

Lauritsen, F.R., L.T. Nielsen, H. Degn, D. Lloyd, and S. Bohatka (1991) Identification of dissolved volatile metabolites in microbial cultures by membrane inlet tandem mass spectrometry. *Biol. Mass Spectrom.* **20**: 253–258.

Leisola, M.S.A., B. Kozulic, F. Meussdoerffer, and A. Fiechter (1987) Homology among multiple extracellular peroxidases from *Phanerochaete chrysosporium. J. Biol. Chem.* **262**: 419–424.

Leonowicz, A., J.M. Sarkar, and J.-M. Bollag (1988) Improvement in the stability and reusability of a fungal laccase by immobilization on porous glass. *Appl. Microbiol. Biotechnol.* **29**: 129–135.

Leontievsky, A.A., N.M. Myasoedova, B.P. Baskunov, C.S. Evans, and L.A. Golovleva (2000) Transformation of 2,4,6-trichlorophenol by the white rot fungi *Panus tigrinus* and *Coriolus versicolor. Biodegradation* **11**: 331–340.

Leontievsky, A.A., N.M. Myasoedova, B.P. Baskunov, L.A. Golovleva, C. Bucke, and C.S. Evans (2001) Transformation of 2,4,6-trichlorophenol by free and immobilized fungal laccase. *Arch. Microbiol. Biotechnol.* **57**: 85–91.

Lestan, D., M. Lestan, J.A. Chapelle, and R.T. Lamar (1996) Biological potential of fungal inocula for bioaugmentation of contaminated soils. *J. Ind. Microbiol.* **16**: 286–294.

Leyva, E., E. Moctezuma, M.G. Ruiz, and L. Torres-Martinez (1998) Photodegradation of phenol and 4-chlorophenol by BaO–Li$_2$O–TiO$_2$ catalysts. *Catal. Today* **40**: 367–376.

Lin, J.E., H.Y. Wang, and R.F. Hickey (1991) Degradation kinetics of pentachlorophenol by *Phanerochaete chrysosporium. Biotechnol. Bioeng.* **38**: 273–279.

Liu, Y., F. Yang, P.L. Yue, and G. Chen (2001) Catalytic dechlorination of chlorophenols in water by palladium/iron. *Water Res.* **35**: 1887–1890.

Logan, B.E., B.C. Alleman, G.L. Amy, and R.L. Gilbertson (1994) Adsorption and removal of pentachlorophenol by white rot fungi in batch cultures. *Water Res.* **28**: 1533–1538.

Loh, K.-C., and S. Ranganath (2005) External-loop fluidized bed airlift bioreactor (EFBAB) for the cometabolic biotransformation of 4-chlorophenol (4-cp) in the presence of phenol. *Chem. Eng. Sci.* **60**: 6313–6319.

Lu, C.-J., C.-M. Lee, and C.-Z. Huang (1996) Biodegradation of chlorophenols by immobilized pure-culture microorganisms. *Water Sci. Technol.* **34**: 67–72.

Luke, A.K., and S.G. Burton (2001) A novel application for *Neurospora crassa*: progress from batch culture to a membrane bioreactor for the bioremediation of phenols. *Enzyme Microb. Technol.* **29**: 348–356.

Lyr, H. (1963) Enzymatische Detoxifikation chlorierter Phenole. *Phytopathol. Z.* **47**: 73–83.

Makkar, R.S., A. Tsuneda, K. Tokuyasu, and Y. Mori (2001) *Lentinula edodes* produces a multicomponent protein complex containing manganese(II)-dependent peroxidase, laccase and β-glucosidase. *FEMS Microbiol. Lett.* **200**: 175–179.

Makower, A., A.V. Eremenko, K. Streffer, U. Wollenberger, and F.W. Scheller (1996) Tyrosinase-glucose dehydrogenase substrate-recycling biosensor. *Biosens. Bioelectron.* **11**: xvii.

Marco, M.-P., S. Chiron, J. Gascon, B.D. Hammock, and D. Barcelon (1995) Validation of two immunoassay methods for environmental monitoring of carbaryl and 1-naphthol in ground water. *Anal. Chim. Acta* **31**: 319–329.

Marko-Varga, G., J. Emneus, L. Gorton, and T. Ruzgas (1995) Development of enzyme-based amperometric sensors for the determination of phenolic compounds. *Trends Anal. Chem.* **14**: 319–328.

Marr, J., S. Kremer, O. Sterner, and H. Anke (1996) Transformation and mineralization of halophenols by *Penicillium simplicissimum* SK9117. *Biodegradation* **7**: 165–171.

Martinez, D., L.F. Larrondo, N. Putnam, et al. (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. *Nat. Biotechnol.* **22**: 695–700.

Matsuzaki, F., and H. Wariishi (2004) Functional diversity of cytochrome P450s of the white-rot fungus *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Commun.* **324**: 387–393.

Mayer, A.M., and R.C. Staples (2002) Laccase: new functions for an old enzyme. *Phytochemistry* **60**: 551–565.

McGrath, R., and I. Singleton (2000) Pentachlorophenol transformation in soil: a toxicological assessment. *Soil Biol. Biochem.* **32**: 1311–1314.

Mehmood, Z., D.E. Kelly, and S.L. Kelly (1997) Cytochrome P450 3A4 mediated metabolism of 2,4-dichlorophenol. *Chemosphere* **34**: 2281–2291.

Middelhoven, W.J. (1993) Catabolism of benzene compounds by ascomycetous and basidiomycetous yeasts and yeastslike fungi: a literature review and an experimental approach. *Antonie Leeuwenhoek* **63**: 125–144.

Middelhoven, W.J., M. Koorevaar, and G.W. Schuur (1992a) Degradation of benzene compounds by yeasts in acidic soils. *Plant Soil* **145**: 37–43.

Middelhoven, W.J., A. Coenen, B. Kraakman, and M.D. Sollewijn Gelpke (1992b) Degradation of some phenols and hydroxybenzoates by the imperfect ascomycetous yeasts *Candida parapsilosis* and *Arxula adeninivorans*: evidence for an operative gentisate pathway. *Antonie Leeuwenhoek* **62**: 181–187.

Mileski, G.J., J.A. Bumpus, M.A. Jurek, and S.D. Aust (1988) Biodegradation of pentachlorophenol by the white-rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **54**: 2885–2889.

Minard, R.D., S.J. Liu, and J.-M. Bollag (1981) Oligomers and quinones from 2,4-dichlorophenol. *J. Agric. Food Chem.* **29**: 250–253.

Morgan, P., S.A. Lee, S.T. Lewis, A.N. Sheppard, and R.J. Watkinson (1993) Growth and biodegradation by white-rot fungi inoculated into soil. *Soil Biol. Biochem.* **25**: 279–287.

Morita, Y., H. Yamashita, B. Mikami, H. Iwamoto, S. Aibara, M. Terada, and J. Minami (1988) Purification, crystallization, and characterization of peroxidase from *Coprinus cinereus*. *J. Biochem.* (*Tokyo*) **103**: 693–699.

Mortberg, M., A. Spanning, and H.Y. Neujahr (1988) Induction of high-affinity phenol uptake in glycerol-grown *Trichosporon cutaneum*. *J. Bacteriol.* **170**: 2383–2384.

Munoz, C., F. Guillen, A.T. Martinez, and M.J. Martinez (1997) Laccase isoenzymes of *Pleurotus eryngii*: characterization, catalytic properties, and participation in activation of molecular oxygen and Mn^{2+} oxidation. *Appl. Environ. Microbiol.* **63**: 2166–2174.

Munteanu, F.-D., A. Lindgren, J. Emneus, L. Gorton, et al. (1998) Bioelectrochemical monitoring of phenols and aromatic amines in flow injection using novel plant peroxidases. *Anal. Chem.* **70**: 2596–2600.

Naish-Byfield, S., and P.A. Riley (1998) Tyrosinase kinetics: failure of acceleration in oxidation of ring-blocked monohydric phenol substrate. *Pigment Cell Res.* **11**: 94–97.

Naish-Byfield, S., C.J. Cooksey, and P.A. Riley (1994) Oxidation of monohydric phenol substrates by tyrosinase: effect of dithiothreitol on kinetics. *Biochem. J.* **304**: 155–162.

Neujahr, H.Y. (1984) Biosensors for environmental control. *Biotechnol. Genet. Eng. Rev.* **1**: 167–185.

Neujahr, H.Y. (1990) Yeasts in biodegradation and biodeterioration process. *Bioprocess Technol.* **5**: 321–348.

Nicell, J.A., L. Al-Kassim, J.K. Bewtra, and K.E. Taylor (1993) Wastewater treatment by enzyme catalyzed polymerization and precipitation. *Biodeterior. Abstr.* **7**: 1–7.

Nistor, C., and J. Emneus (1999) Bioanalytical tools for monitoring polar pollutants. *Waste Manag.* **19**: 147–170.

Okeke, B.C., A. Paterson, J.E. Smith, and T.A. Watson-Craik (1997) Comparative biotransformation of pentachlorophenol in soils by solid substrate cultures of *Lentinula edodes. Appl. Microbiol. Biotechnol.* **48**: 563–569.

Onnerfjord, P., J. Emneus, G. Marko-Varga, and L. Gorton (1995) Tyrosinase graphite-epoxy based composite electrodes for detection of phenols. *Biosens. Bioelectron.* **10**: 607–619.

Orser, C.S., L. Xun, and C. Lange (1997) Genes and enzymes involved in the microbial degradation of pentachlorophenol. *Biotechnol. Adv.* **15**: 213.

Palmieri, G., P. Giardina, L. Marzullo, B. Desiderio, G. Nittii, R. Cannio, and G. Sannia (1993) Stability and activity of a phenol oxidase from the ligninolytic fungus *Pleurotus ostreatus. Appl. Microbiol. Biotechnol.* **39**: 632–636.

Palmieri, G., P. Giardina, C. Bianco, A. Scaloni, A. Capasso, and G. Sannia (1997) A novel white laccase from *Pleurotus ostreatus. J. Biol. Chem.* **272**: 31301–31307.

Paszczynski, A., V.-B. Huynh, and R.L. Crawford (1986) Comparison of ligninase-I and peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* **244**: 750–765.

Paxeus, N. (1996) Organic pollutants in the effluents of large wastewater treatment plants in Sweden. *Water Res.* **30**: 1115–1122.

Perez, R.B., G.G. Benito, and M.P. Miranda (1997) Chlorophenol degradation by *Phanerochaete chrysosporium. Bioresour. Technol.* **60**: 207–213.

Petersen, J.F.W., J.W. Tams, J. Vind, A. Svensson, et al. (1993) Crystallization and x-ray diffraction analysis of recombinant *Coprinus cinereus* peroxidase. *J. Mol. Biol.* **232**: 989–991.

Polnisch, E., H. Kneifel, H. Franzke, and K.H. Hofmann (1992) Degradation and dehalogenation of monochlorophenols by the phenol-assimilating yeast *Candida maltosa. Biodegradation* **2**: 193–199.

Puhakka, J.A., E.S. Melin, K.T. Jarvinen, P.M. Kora, et al. (1995) Fluidized-bed biofilms for chlorophenol mineralization. *Water Sci. Technol.* **31**: 227–235.

Rahouti, M., J.-L. Benoit-Guyod, F. Seigle-Murandi, and P. Guiraud (1995) Sensitivity and specificity of phenol oxidase reactions of 1059 strains and species of Micromycetes cultivated on malt/agar medium. *World J. Microbiol. Biotechnol.* **11**: 497–501.

Rahouti, M., R. Steiman, F. Seigle-Murandi, and L.P. Christov (1999) Growth of 1044 strains and species of fungi on 7 phenolic lignin model compounds. *Chemosphere* **38**: 2549–2559.

Reddy, G.V.B., and M.H. Gold (2000) Degradation of pentachlorophenol by *Phanerochaete chrysosporium*: intermediates and reactions involved. *Microbiology* **146**: 405–413.

Reddy, G.V.B., M.D. Sollewijn Gelpke, and M.H. Gold (1998) Degradation of 2,4,6-trichlorophenol by *Phanerochaete chrysosporium*: involvement of reductive dechlorination. *J. Bacteriol.* **180**: 5159–5164.

Richter, D.L., J.I. Warner, and A.L. Stephens (2003) A comparison of mycorrhizal and saprophytic fungus tolerance to creosote in vitro. *Int. Biodeterior. Biodegrad.* **51**: 195–202.

Ricotta, A., R.F. Unz, and J.-M. Bollag (1996) Role of laccase in the degradation of pentachlorophenol. *Bull. Environ. Contam. Toxicol.* **57**: 560–567.

Ricoux, R., J.L. Boucher, D. Mansuy, and J.P. Mahy (2001) Microperoxidase 8 catalyzed nitration of phenol by nitrogen dioxide radicals. *Eur. J. Biochem./FEBS* **268**: 3783–3788.

Rodriguez, E., O. Nuero, F. Guillen, A.T. Martinez, and M.J. Martinez (2004) Degradation of phenolic and non-phenolic aromatic pollutants by four *Pleurotus* species: the role of laccase and versatile peroxidase. *Soil Biol. Biochem.* **36**: 909–916.

Rodriguez-Delgado, M.A., S. Malovana, J.P. Perez, T. Borges, and F.J. Garcia Montelongo (2001) Separation of phenolic compounds by high-performance liquid chromatography with absorbance and fluorimetric detection. *J. Chromatogr. A* **912**: 249–257.

Roper, J.C., J.M. Sarkar, J. Dec, and J.-M. Bollag (1995) Enhanced enzymatic removal of chlorophenols in the presence of co-substrates. *Water Res.* **29**: 2720–2724.

Ros, J.R., J.N. Rodriguez-Lopez, R. Varon, and F. Garcia-Canovas (1994) Kinetic study of the oxidation of 4-*tert*-butylphenol by tyrosinase. *Eur. J. Biochem./FEBS* **222**: 449–452.

Ruggeiro, P., J.M. Sarkar, and J.-M. Bollag (1989) Detoxification of 2,4-dichlorophenol by a laccase immobilized on soil or clay. *Soil Sci.* **14**: 361–370.

Ruttimann-Johnson, C., and R.T. Lamar (1997) Binding of pentachlorophenol to humic substances in soil by the action of white rot fungi. *Soil Biol. Biochem.* **29**: 1143–1148.

Ruzgas, T., J. Emneus, L. Gorton, and G. Marko-Varga (1995) The development of a peroxidase biosensor for monitoring phenol and related aromatic compounds. *Anal. Chim. Acta* **311**: 245–253.

Saito, T., K. Kato, Y. Yokogawa, M. Nishida, and N. Yamashita (2004) Detoxification of bisphenol A and nonylphenol by purified extracellular laccase from a fungus isolated from soil. *J. Biosci. Bioeng.* **98**: 64–66.

Sampaio, J.P. (1999) Utilization of low molecular weight aromatic compounds by heterobasidiomycetous yeasts: taxonomic implications. *Can. J. Microbiol.* **45**: 491–512.

Santos, V.L., and V.R. Linardi (2004) Biodegradation of phenol by a filamentous fungi isolated from industrial effluents: identification and degradation potential. *Process Biochem.* **39**: 1001–1006.

Sarakha, M., M. Bolte, and H.D. Burrows (1997) The photo-oxidation of 2,6-dimethylphenol and monophenylphenols by uranyl ion in aqueous solution. *J. Photochem. Photobiol. A* **107**: 101–106.

Sayre, L.M., and D.V. Nadkarni (1994) Direct conversion of phenols to *o*-quinones by copper(I) dioxygen: questions regarding the monophenolase activity of tyrosine mimics. *J. Am. Chem. Soc.* **116**: 3157–3158.

Seeholzer-Nguyen, B., and B. Hock (1991) Biodegradation of trichlorophenols by fungi. *Angew. Bot.* **65**: 219–227.

Seigle-Murandi, F., R. Steiman, J.-L. Benoit-Guyod, and P. Guiraud (1992) Fungal degradation of pentachlorophenol by micromycetes. I. Zygomycetes. *Environ. Toxicol. Water Qual.* **7**: 125–139.

Seigle-Murandi, F., R. Steiman, J.-L. Benoit-Guyod, and P. Guiraud (1993) Fungal degradation of pentachlorophenol by micromycetes. *J. Biotechnol.* **30**: 27–35.

Seigle-Murandi, F., A. Toe, J.-L. Benoit-Guyod, R. Steiman, and M. Kadri (1995) Depletion of pentachlorophenol by Deuteromycetes isolated from soil. *Chemosphere* **31**: 2677–2686.

Semple, K.T., and R.B. Cain (1996) Biodegradation of phenols by the alga *Ochromonas danica. Appl. Environ. Microbiol.* **62**: 1265–1273.

Semple, K.T., and R.B. Cain (1997) Degradation of phenol and its methylated homologues by *Ochromonas danica. FEMS Microbiol. Lett.* **152**: 133–139.

Semple, K.T., B.J. Reid, and T.R. Fermor (2001) Impact of composting strategies on the treatment of soils contaminated with organic pollutants. *Environ. Pollut.* **112**: 269–283.

Setti, L., S. Giuliani, G. Spinozzi, and P.G. Pifferi (1999) Laccase catalyzed-oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone and methoxyphenols. *Enzyme Microb. Technol.* **25**: 285–289.

Shim, S.-S., and K. Kawamoto (2002) Enzyme production activity of *Phanerochaete chrysosporium* and degradation of pentachlorophenol in a bioreactor. *Water Res.* **36**: 4445–4454.

Shuttleworth, K.L., and J.M. Bollag (1986) Soluble and immobilized laccase as catalysts for the transformation of substituted phenols. *Enzyme Microb. Technol.* **8**: 171–177.

Siddique, M.H., C.C. St. Pierre, N. Biswas, J.K. Bewtra, and K.E. Taylor (1993) Immobilized enzyme catalyzed removal of 4-chlorophenol from aqueous solution. *Water Res.* **27**: 883–890.

Skladal, P. (1991) Mushroom tyrosinase-modified carbon paste electrode as an amperometric biosensor for phenols. *Collect. Czech. Chem. Commun.* **56**: 1427–1433.

Smulevich, G., F. Neri, M.P. Marzocchi, and K.G. Welinder (1996) Versatility of heme coordination demonstrated in a fungal peroxidase: absorption and resonance Raman studies of *Coprinus cinereus* peroxidase and the Asp245→Asn mutant at various pH values. *Biochemistry* **35**: 10576–10585.

Spanning, A., and H.Y. Neujahr (1990) The effect of glucose on enzyme activities and phenol utilization in *Trichosporon cutaneum* grown in continuous culture. *J. Gen. Microbiol.* **136**: 1491–1495.

Spivack, J., T.K. Leib, and J.H. Lobos (1994) Novel pathway for bacterial metabolism of bisphenol A. *J. Biol. Chem*. **269**: 7323–7329.

Steiman, R., J.-L. Benoit-Guyod, F. Seigle-Murandi, L. Sage, and A. Toe (1994) Biodegradation of pentachlorophenol by micromycetes. II. Ascomycetes, basidiomycetes, and yeasts. *Environ. Toxicol. Water Qual.* **9**: 1–6.

Sugumaran, M., and J.L. Bolton (1998) Laccase—and not tyrosinase—is the enzyme responsible for quinone methide production from 2,6-dimethoxy-4-allyl phenol. *Arch. Biochem. Biophys.* **353**: 207–212.

Sun, W.-Q., and G.F. Payne (1996) Tyrosinase-containing chitosan gels: a combined catalyst and sorbent for selective phenol removal. *Biotechnol. Bioeng.* **51**: 79–86.

Sykora, J., M. Pado, M. Tatarko, and M. Izakovic (1997) Homogeneous photo-oxidation of phenols: influence of metals. *J. Photochem. Photobiol. A* **110**: 167–175.

Tartakovsky, B., M.F. Manuel, D. Beaumier, C.W. Greer, and S.R. Guiot (2001) Enhanced selection of an anaerobic pentachlorophenol-degrading consortium. *Biotechnol. Bioeng.* **73**: 476–483.

Taseli, B.K., and C.F. Gokcay (2005) Degradation of chlorinated compounds by *Penicillium camemberti* in batch and up-flow column reactors. *Process Biochem.* **40**: 917–923.

Tayal, A.K., L. Das, and I. Kaur (1999) Biodegradation of pentachlorophenol (PCP) by white rot fungal strains screened from local sources and its estimation by high-performance liquid chromatography. *Biomed. Chromatogr.* **13**: 220–224.

Tikoo, V., A.H. Scragg, and S.W. Shales (1996) Microalgal biodegradation of pentachlorophenol. *Int. Biodeterior. Biodegrad.* **37**: 247.

Tomasi, I., I. Artaud, Y. Bertheau, and D. Mansuy (1995) Metabolism of polychlorinated phenols by *Pseudomonas cepacia* AC1100: determination of the first two steps and specific inhibitory effect of methimazole. *J. Bacteriol.* **177**: 307–311.

Torres, T.Y., and J.P. Rosazza (2001) Microbial transformations of *p*-coumaric acid by *Bacillus megaterium* and *Curvularia lunata*. *J. Nat. Prod.* **64**: 1408–1414.

Trejo-Hernandez, M.R., A. Lopez-Munguia, and R. Quintero Ramirez (2001) Residual compost of *Agaricus bisporus* as a source of crude laccase for enzymic oxidation of phenolic compounds. *Process Biochem.* **36**: 635–639.

Tsutsumi, Y., T. Haneda, and T. Nishida (2001) Removal of estrogenic activities of bisphenol A and nonylphenol by oxidative enzymes from lignin-degrading basidiomycetes. *Chemosphere* **42**: 271–276.

Uchida, H., T. Fukuda, H. Miyamoto, T. Kawabata, et al. (2001) Polymerization of bisphenol A by purified laccase from *Trametes villosa*. *Biochem. Biophys. Res. Commun.* **287**: 355–358.

Ullah, M.A., C.T. Bedford, and C.S. Evans (2000a) Reactions of pentachlorophenol with laccase from *Coriolus versicolor*. *Appl. Microbiol. Biotechnol.* **53**: 230–234.

Ullah, M.A., H. Kadhim, R.A. Rastall, and C.S. Evans (2000b) Evaluation of solid substrates for enzyme production by *Coriolus versicolor*, for use in bioremediation of chlorophenols in aqueous effluents. *Appl. Microbiol. Biotechnol.* **54**: 832–837.

Upadhyay, R.C., and M. Hofrichter (1993) Effect of phenol on the mycelial growth and fructification in some of basidiomycetous fungi. *J. Basic Microbiol.* **33**: 343–347.

USEPA (1979) Phenol. In: *Ambient Water Criteria*. PB 296,787. Office of Water Planning and Standards, U.S. Environmental Protection Agency, Washington, DC.

USEPA (2000) Preliminary remediation goals (PRGs) for Superfund and RCRA programs. www.epa.gov/region09/waste/sfund/prg/faq.htm.

Valli, K., and M.H. Gold (1991) Degradation of 2,4-dichlorophenol by the lignin-degrading fungus *Phanerochaete chrysosporium*. *J. Bacteriol*. **173**: 345–352.

Vallini, G., S. Grassinetti, and G. Scorzetti (1997) *Candida aquaetextoris* sp. nov., a new species of yeast occurring in sludge from a textile industry wastewater treatment plant in Tuscany, Italy. *Int. J. Syst. Bacteriol.* **47**: 336–340.

Vallini, G., S. Grassinetti, F.D. Andrea, G. Catelani, and M. Agnolucci (2001) Biodegradation of 4-(1-nonyl)phenol by axenic cultures of the yeast *Candida aquaetextoris*: identification of microbial breakdown products and proposal of a possible metabolic pathway. *Int. Biodeterior. Biodegrad.* **47**: 133–140.

van Gelder, C.W.G., W.A. Flurkey, and H.J. Wichers (1997) Sequence and structural features of plant and fungal tyrosinases. *Phytochemistry* **45**: 1309–1323.

van Schie, P.M., and L.Y. Young (2000) Biodegradation of phenol: mechanisms and applications. *Bioremed. J.* **4**: 1–18.

Veitch, N.C., J.W. Tams, J. Vind, H. Dalboge, and K.G. Welinder (1994) NMR studies of recombinant *Coprinus* peroxidase and three site-directed mutants: implications for peroxidase substrate binding. *Eur. J. Biochem.* **22**: 909–918.

Veitch, N.C., Y. Gao, and K.G. Welinder (1996) The Asp245→Asn mutant of *Coprinus cinereus* peroxidase: characterization by ^{1}H-NMR spectroscopy and comparison with the wild-type enzyme. *Biochemistry* **35**: 14370–14380.

Ventura, F., M.R. Boleda, R. Lloret, and J. Martin-Alonso (1998) Strategies for the identification of compounds causing odours in water: a study of creosote spills. *Water Res.* **32**: 503–509.

Vione, D., V. Maurino, C. Minero, and E. Pelizzetti (2001a) Phenol photonitration upon UV irradiation of nitrite in aqueous solution. I. Effects of oxygen and 2-propanol. *Chemosphere* **45**: 893–902.

Vione, D., V. Maurino, C. Minero, and E. Pelizzetti (2001b) Phenol photonitration upon UV irradiation of nitrite in aqueous solution. I. Effects of pH and TiO_2. *Chemosphere* **45**: 903–910.

Vipulanandan, C., S. Wang, and S. Krishnan (1994) Biodegradation of phenol. In: *Remediation of Hazardous Waste Contaminated Soils*, D.L. Wise and D.J. Trentolo, eds. Marcel Dekker, New York, pp. 199–225.

Vroumsia, T., R. Steiman, F. Seigle-Murandi, and J.-L. Benoit-Guyod (2005) Fungal bioconversion of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-dichlorophenol (2,4-DCP). *Chemosphere* **60**: 1471–1480.

Wada, S., H. Ichikawa, and K. Tatsumi (1993) Removal of phenols from wastewater by soluble and immobilized tyrosine. *Biotechnol. Bioeng.* **42**: 854–858.

Wada, S., H. Ichikawa, and K. Tatsumi (1995) Removal of phenols and aromatic amines from wastewater by a combination treatment with tyrosinase and a coagulant. *Biotechnol. Bioeng.* **45**: 304–309.

Walter, N., K. Boyd-Wilson, L. Boul, et al. (2005) Field-scale bioremediation of pentachlorophenol by *Trametes versicolor*. *Int. Biodeterior. Biodegrad*. **56**: 51–57.

Wang, J., and Q. Chen (1995) Microfabricated phenol biosensors based on screen printing of tyrosinase containing carbon ink. *Anal. Lett.* **28**: 1131–1142.

Wannstedt, C., D. Rotella, and J.F. Siuda (1990) Chloroperoxidase mediated halogenation of phenols. *Bull. Environ. Contam. Toxicol.* **44**: 282–287.

Ward, G., Y. Hadar, I. Bilkis, L. Konstantinovsky, and C.G. Dosoretz (2001) Initial steps of ferulic acid polymerization by lignin peroxidase. *J. Biol. Chem.* **276**: 18734–18741.

Webb, M.D., G. Ewbank, J. Perkins, and A.J. Mccarthy (2001) Metabolism of pentachlorophenol by *Saccharomonospora viridis* strains isolated from mushroom compost. *Soil Biol. Biochem.* **33**: 1903–1916.

Weitz, H.J., C.D. Campbell, and K. Killham (2002) Development of a novel, bioluminescence-based, fungal bioassay for toxicity testing. *Environ. Microbiol.* **4**: 422–429.

Wetzstein, H.-G., N. Schmeer, and W. Karl (1997) Degradation of the fluoroquinolone Enrofloxacin by the brown rot fungus *Gloeophyllum striatum*: identification of metabolites. *Appl. Environ. Microbiol.* **63**: 4272–4281.

Wilcox, D.E., A.G. Porras, Y.T. Hwang, K. Lerch, M.E. Winkler, and E.I. Solomon (1985) Substrate analog binding to the coupled binuclear copper active site in tyrosinase. *J. Am. Chem. Soc.* **107**: 4015–4027.

Wollenberger, U., B. Neumann, K. Riedel, and F.W. Scheller (1994) Enzyme and microbial sensors for phosphate, phenols, pesticides and peroxides. *Fresenius' J. Anal. Chem.* **348**: 563–566.

Wu, F., H. Ozaki, Y. Terashima, T. Imada, and Y. Ohkouchi (1996) Activities of ligninolytic enzymes of the white rot fungus, *Phanerochaete chrysosporium* and its recalcitrant substance degradability. *Water Sci. Technol.* **34**: 69–78.

Wu, C., X. Liu, D. Wei, J. Fan, and L. Wang (2001a) Photosonochemical degradation of phenol in water. *Water Res.* **35**: 3927–3933.

Wu, Q., X. Hu, P.L. Yue, X.S. Zhao, and G.Q. Lu (2001b) Copper/MCM-41 as catalyst for the wet oxidation of phenol. *Appl. Catal. B* **32**: 151–156.

Yan, J., W. Jianping, L. Hongmei, Y. Suliang, and H. Zongding (2005) The biodegradation of phenol at high initial concentration by the yeast *Candida tropicalis*. *Biochem. Eng. J.* **24**: 243–247.

Yaropolov, A.I., A.N. Kharybin, J. Emneus, G. Marko-Varga, and L. Gorton (1995) Flow-injection analysis of phenols at a graphite electrode modified with co-immobilized laccase and tyrosinase. *Anal. Chim. Acta* **308**: 137–144.

Yu, J., and P.E. Savage (2000) Phenol oxidation over CuO/Al_2O_3 in supercritical water. *Appl. Catal. B* **28**: 275–288.

Zavarzina, A.G., A.A. Leontievsky, L.A. Golovleva, and S.Y. Trofimov (2004) Biotransformation of soil humic acids by blue laccase of *Panus tigrinus* 8/18: an in vitro study. *Soil Biol. Biochem.* **36**: 359–369.

Zouari, H., M. Labat, and S. Sayadi (2002) Degradation of 4-chlorophenol by the white rot fungus *Phanerochaete chrysosporium* in free and immobilized cultures. *Bioresour. Technol.* **84**: 145–150.

8

FUNGAL METABOLISM OF POLYCYCLIC AROMATIC HYDROCARBONS

8.1 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a class of ubiquitous xenobiotic environmental pollutants that have been detected in several aquatic and terrestrial ecosystems. PAHs are lipophilic constituents that are released into the environment as a result of a variety of activities, such as incomplete combustion of fossil fuels, shale oil, and cigarette smoke; accidental discharge of petroleum or during the use and disposal of petroleum products; and coal gasification and liquefaction (IARC, 1983). PAHs are also produced due to the incineration of refuse and wastes and burning of agricultural and forest residues. The PAH constituents adhere to suspended particles and settle down in soils and sediments of rivers and estuaries.

PAHs consist of three or more benzene rings fused in linear, angular, or cluster arrangements. They are thermodynamically stable, due to strong negative resonance energy. Due to their hydrophobic nature and low vapor pressure, they tend to adsorb and accumulate in sediments. Hydrophobicity increases and volatility decreases with the number of fused benzene rings. The possible fates of PAH compounds in soils are volatilization, photooxidation, chemical oxidation, adsorption, adhesion, and bioaccumulation. Significant loss of PAHs with more than three rings in soils does not happen abiotically. High-molecular-weight PAHs are more recalcitrant than low-molecular-weight PAHs to microbial attack, and this may be attributed to

bioavailability, nutrients, redox potential or other limiting factors. Moreover, the half-lives of five- or six-ring PAHs are estimated to be on the order of years in various ecosystems.

Based on the structure and mechanism of activation, many PAHs exhibit toxic, mutagenic, tumorigenic, and carcinogenic properties. Several studies have indicated the acute toxicity of low-molecular-weight PAHs and genotoxicity of higher-molecular-weight PAHs. Metabolic activation of PAHs to electrophilic species forms covalent binding with nucleophilic groups of deoxyribonucleic acid (DNA), thereby causing mutations. Due to their persistence in the environment and genotoxicity, PAHs represent a significant health risk to humans. Thus, 16 PAHs have been regulated as priority pollutants in aquatic and terrestrial ecosystems by the U.S. Environmental Protection Agency (Keith and Telliard, 1979). Various approaches have been adopted to establish cleanup criteria in the United States, Canada, European Community, and Scandinavia (Siegrist, 1990; Wilson and Jones, 1993).

Improper disposal methods and inadequate control of these materials have created widespread contamination in soils, groundwater, and surface water bodies. It is difficult to achieve cleanup at the sites because some remediation technologies are not acceptable to the public or may not be amenable to particular sites. A wide variety of physical, chemical, and biological methods have been developed for the treatment of such recalcitrant wastes, but some of these are very expensive. Commonly used strategies of excavation, followed by incineration, and/or landfilling are now less environmentally acceptable. Landfarming has also been employed at less contaminated sites. Since passage of the 1984 Hazardous and Solid Waste Amendments (HSWA), landfarming of such wastes has ceased (Arbuckle et al., 1991). Hence, interest has increased in cleaning up the sites by bioremediation. At present, bioremediation is effective for soils contaminated with low-molecular-weight PAHs.

8.2   OCCURRENCE OF PAHs IN THE ENVIRONMENT

PAHs are widely distributed in the natural environment. They have been detected in a wide variety of air, soil, and sediment samples. Contamination by PAHs can be found in foods. PAHs from atmosphere deposition may also accumulate in plants, which may result in human exposure through food consumption. PAHs are also found in creosote (about 85% by weight) and anthracene oil, which are commonly used to treat wood. Motor vehicle emissions contribute PAH pollution to the air through exhaust condensate and particulates, tire particles, and lubricating oils and greases. Sources of PAH contamination in sediments include atmospheric deposition, marine seeps of petroleum, and offshore production or petroleum transportation. In industrial countries, anthropogenic combustion activities, the main source of PAHs in soils, have increased soil PAH concentration over the last 100 to 150 years. Industrial activities are also associated with the production, processing, use,

and disposal of PAH-containing materials. The leaves of *Quercus ilex* L. can be used to monitor the degree of PAHs in the air (Alfani et al., 2001).

The concentration of PAHs in contaminated soils varies depending on the industrial activity at a site. Contamination by PAHs is well established in soils, sediments, and groundwater at different facilities, such as creosote production and wood preserving, gas works and manufacturing gas plants, and petrochemical and Superfund sites. Various processes are also known to control bioaccumulation of PAHs in marine organisms (Meador et al., 1995). The release of PAHs in the air, soils and sediments, marine organisms and plants has been discussed by Juhasz and Naidu (2000).

8.3 ALTERNATIVE PAH METABOLISM

Effective PAH biodegradation in soils or water is a function of their physical and chemical properties, concentrations, rates of diffusion, and bioavailability. It also depends on soil type, moisture content, presence of nutrients, redox conditions, pH, temperature, sediment toxicity, seasonal factors, PAH-degrading microbes, and other factors. PAH metabolism by microorganisms has been a topic of great interest to several researchers (Cerniglia, 1993; Sutherland et al., 1995; Pothuluri and Cerniglia, 1998; Juhasz and Naidu, 2000).

8.3.1 Bacteria

At present, numerous genera of bacteria have been shown to oxidize PAHs. A great diversity of bacteria are known to metabolize low-molecular-weight PAHs, and a few genera are recognized as degrading high-molecular-weight PAHs. Several researchers have shown the role of various species of *Rhodococcus*, *Mycobacterium*, *Alcaligenes*, *Pseudomonas*, *Beijerinckia*, *Staphylococcus*, *Arthrobacter*, *Nocardia*, and *Gordona* in the degradation of high-molecular-weight PAHs. Recently, Cerniglia (2003) discussed the bacterial degradation of PAHs, including the metabolism, mechanism of oxidation, and genetic analysis of *Mycobacterium* sp. PYR-1.

In the majority of bacteria, an aerobic pathway for the degradation of PAHs involves oxidation of the benzene ring by dioxygenases to form *cis*-dihydrodiols, as shown in Figure 8.1 (Cerniglia and Sutherland, 2001). These dihydrodiols are converted to diphenols that are subsequently cleaved by other dioxygenases, and further catabolism results in the formation of tricarboxylic acid intermediates. A few bacteria catalyze the degradation of PAHs to *trans*-dihydrodiols. Little is known of the metabolism of PAHs by sulfate-reducing and methane-oxidizing bacteria. PAHs can also be degraded by aerobic mixed bacteria.

Bacterial metabolism of naphthalene, anthracene, phenanthrene, and pyrene has been studied extensively. The mean rate of phenanthrene degradation has been shown to occur due to a sole source of carbon by *Beijerinckia*
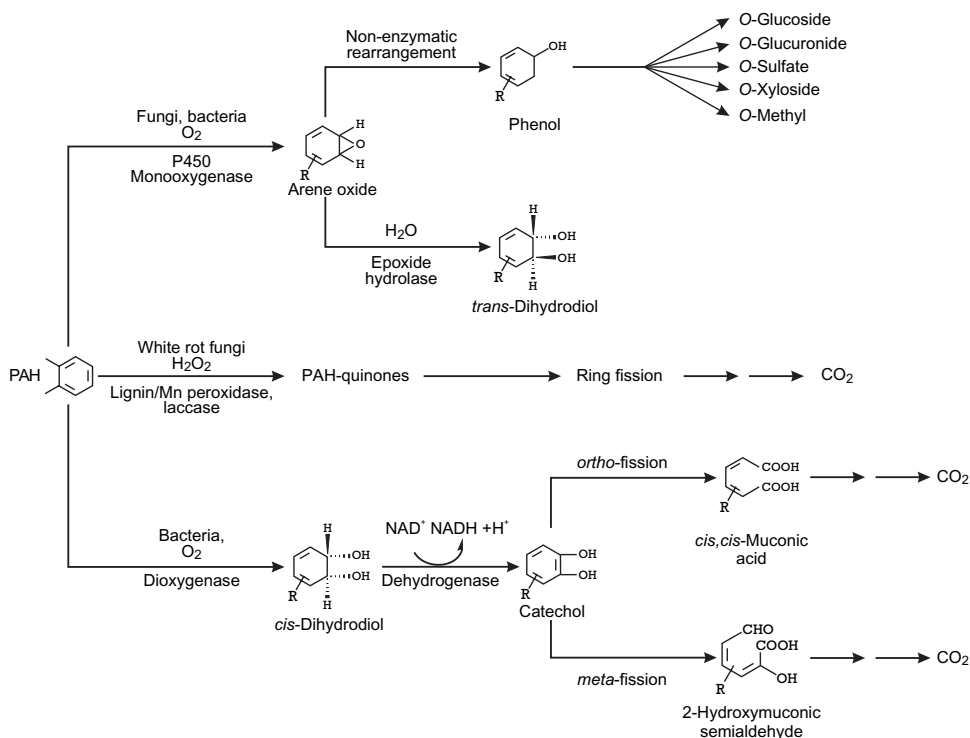
Figure 8.1   Initial steps in the pathways of degradation of polycyclic aromatic hydrocarbons by fungi and bacteria. [Reprinted from Cerniglia and Sutherland (2001), copyright © with permission from Cambridge University Press. Also, adapted from Cerniglia (1993), copyright © with permission from Elsevier.]

mobilis 1f (Surovtseva et al., 1999). The strain also grows in a mineral medium with creosote as the sole source of carbon. In *Comamonas testosteroni*, less homologous group of genes coding for enzymes have been found for the degradation of PAHs (Goyal and Zylstra, 1996). Four structural genes and two putative promoters have been identified for the utilization of naphthalene, phenanthrene, and fluoranthene in *Sphingomonas paucimobilis* var. EPA505 (Story et al., 2000). *Bacillus megaterium* CYP102 mutants exhibit a potential for the preparation of novel PAH bioremediation systems (Carmichael and Wong, 2001). A pathway for the metabolism of pyrene by *Mycobacterium* sp. strain KR2 has also been proposed (Rehmann et al., 1998) that correlates well with pathways identified earlier. A new metabolite was identified during degradation of pyrene by *Mycobacterium* sp. strain AP1, which demonstrates a new branch in the pathway, involving cleavage of both central rings (Vila et al., 2001).

During the last decade, bacteria were also recognized to degrade benzo[*a*]pyrene. Pathways for the degradation of benzo[*a*]pyrene by *Myco-*

bacterium sp. strain RJGII-135 by a dioxygenase system have been proposed (Schneider et al., 1996). Five metabolites have been identified during degradation of fluoranthene by *Mycobacterium* sp. strain KR20 (Rehmann et al., 2001). Bacterial, fungal, and algal benzo[*a*]pyrene metabolism, including the initial concentrations, percentage removal, evolution of CO_2, time of incubation, and production of metabolites, have been reviewed by Juhasz and Naidu (2000).

8.3.2 Algae, Cyanobacteria, and Higher Plants

Algae and cyanobacteria were first shown to degrade PAHs in 1980. A mechanism of oxidation of PAHs by algae is shown by Cerniglia (1993). The algal and cyanobacterial metabolism of PAHs have been discussed by some researchers (Sutherland et al., 1995; Juhasz and Naidu, 2000). Green, red, and brown algae and cyanobacteria oxidized naphthalene to 1-naphthol with minor amounts of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene and 4-hydroxy-1-tetralone (Cerniglia et al., 1980a). A marine cyanobacterium, *Agmenellum quadruplicatum* PR-6, transformed phenenthrene to *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene and 1-methoxyphenanthrene as major metabolites (Narro et al., 1992a). Naphthalene degraded to 1-naphthol via an arene oxide intermediate by another marine bacterium, *Oscillatoria* sp. strain JCM (Narro et al., 1992b). However, enzymes catalyzing the oxidation of PAHs by cyanobacteria are not known at present. Benzo[*a*]pyrene has been completely metabolized to dihydrodiols under golden and white light by the green algae *Selenastrum capricornutum*, *Scenedesmus acutus*, and *Ankistrodesmus braunii* (Warshawsky et al., 1995). The formation of *cis*-dihydrodiols indicates a dioxygenase-catalyzed reaction similar to that of bacteria rather than the monooxygenase-catalyzed reaction that occurs in cyanobacteria, fungi, and mammals.

Several different classes of plants are known to degrade or dissipate or remove PAHs in soil and water. The rhizospheres of nine plant species removed a significant amount of pyrene after 56 days (Liste and Alexander, 2000). Seeding field plots with sorghum (*Sorghum bicolor*), ryegrass (*Lolium perenne*), or St. Augustine grass (*Stenotaphrum secundatum*) enhanced the removal of TPHs and PAHs over three growing seasons (Nedunuri et al., 2000). A multiprocess phytoremediation system comprised of volatilization, photooxidation, microbial remediation, and phytoremediation proved twice as effective as landfarming, 50% more than bioremediation alone, and 45% more than phytoremediation by itself for the removal of 16 priority PAHs over a 4-month period (Huang et al., 2004).

8.4 FUNGAL METABOLISM OF PAHs

Despite the ongoing research during the last two decades, knowledge of the fungal metabolism of PAHs is limited compared to that of bacteria. However,

fungi are as important as bacteria in the bioremediation of PAHs in aquatic and terrestrial environments. Unlike bacteria, fungi do not assimilate PAHs as the sole sources of carbon and energy, but require cometabolite to detoxify them (Wunder et al., 1994; Pothuluri et al., 1995; Casillas et al., 1996). In general, fungi are slow and less efficient than bacteria in PAH degradation. Bacteria are unable to degrade efficiently PAHs that have more than four aromatic rings, whereas fungi can degrade and mineralize PAHs with more than four aromatic rings. Oxidation of PAHs as a prelude to ring fission and assimilation is known in bacteria, whereas fungi hydroxylate PAHs as a prelude to detoxification. Fungi play a significant ecological role, as their polar and reactive metabolites can be mineralized or detoxified to innocuous compounds by indigenous soil bacteria. Moreover, fungal mycelium has the ability to grow into the soil and be distributed through the solid matrix to metabolize PAHs. As a result, fungi can also form bound residues of PAHs in the soil, thereby reducing its toxicity.

A diverse group of fungi has been demonstrated to oxidize PAHs ranging from two to six aromatic rings. Fungal species that have demonstrated significant potential to metabolize PAHs are the Zygomycete *Cunninghamella elegans*, the Ascomycetes *Aspergillus niger* and *Penicillium* sp., and the white-rot Basidiomycetes *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus*, and *Bjerkandera* sp. Nonbasidiomycete fungi oxidize PAHs rather than mineralizing during the initial metabolism. Some white-rot Basidiomycetes have the ability to cleavage benzene rings and mineralize PAHs. The genera of fungi from various ecological groups are also able to degrade PAHs.

In general, filamentous fungi and yeasts have shown oxidative transformation of PAHs to *trans*-dihydrodiols, dihydrodiol epoxides, quinones, and phenols (phase I mechanism). Conjugative products (phase II mechanism) such as glucuronides, glucosides, xylosides, and sulfates are also produced. Conjugation products are nonmutagenic, whereas oxidative products are toxic and bioactive. Arene oxides are unstable intermediates in the formation of corresponding metabolites and have not been isolated from fungal or bacterial cultures. Pathways for the fungal metabolism of PAHs are shown in Figure 8.1 (Cerniglia and Sutherland, 2001). These involve several enzymes, such as intracellular cytochrome P450 and extracellular lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. The formation of hydroxylated metabolites is noteworthy for bioremediation, as it increases the mineralization of these compounds. Fungal metabolism of PAHs has been discussed by several researchers (Cerniglia et al., 1992; Cerniglia, 1997; Kremer and Anke, 1997; Pothuluri and Cerniglia, 1998; Juhasz and Naidu, 2000; Cerniglia and Sutherland, 2001). Yeast metabolism of PAHs has also been detailed (Cerniglia and Crow, 1981; MacGillivray and Shiaris, 1993). Several articles have been written on the biotransformation of several PAHs by the Zygomycete *Cunninghamella elegans* under Cerniglia's leadership. Table 8.1 summarizes the fungal degradation of various types of PAHs and metabolic products.

TABLE 8.1 Fungal Degradation of Polycyclic Aromatic Hydrocarbons and Metabolic Products

Fungus	PAH Conc.	Medium	Degradation/ Mineralization Rate (%)	Metabolic Products	Pathway	Duration (hours/ days/ weeks)	Reference
Cunninghamella elegans	Acenaphthene, 20 mg	Sabouraud dextrose broth	64	1-Acenaphthenol (2.4%), 1,5-dihydroxyacenaphthene (2.7%), *cis*- (1.8%) and *trans*-1, 2-dihydroxyacenaphthene (10.3%),1-acenaphthenone (2.1%), 1, 2-acenaphthenedione (19.9), 6-hydroxyacenaphthenone (24.8%)	1	3 d	Pothuluri et al., 1992b
Pleurotus ostreatus	Anthracene	Basidiomycetes Rich medium	60	9,10-Anthraquinone		3 d	Schutzendubel et al., 1999
	Phenanthrene		12			2 d	
	Fluorene		42	9-Fluorenone		7 d	
	Fluoranthene		12			7 d	
	Pyrene		10			7 d	
Bjerkandera adusta	Anthracene, 0.5 mg		38	9,10-Anthraquinone		3 d	
	Phenanthrene, 0.5 mg		30			7 d	
	Fluorene, 0.5 mg		55	9-Fluorenone		7 d	
	Fluoranthene, 0.5 mg		27			7 d	
	Pyrene, 0.5 mg		28			7 d	
P. ostreatus	Anthracene, 2.5 mg nonlabeled and 1 μCi labeled	Basidiomycetes Rich medium	74	Anthracene *trans*-1, 2-dihydrodiol, 9,10-anthraquinone	1	21 d	Bezalel et al., 1996c
	Fluorene, 2.5 mg nonlabeled and 1 μCi labeled		96	9-Fluorenol, 9-fluorenone	1		
	Pyrene, 2.5 mg nonlabeled and 1 μCi labeled		91	Pyrene *trans*-4,5-dihydrodiol	1		

TABLE 8.1 *Continued*

Fungus	PAH Conc.	Medium	Degradation/ Mineralization Rate (%)	Metabolic Products	Pathway	Duration (hours/ days/ weeks)	Reference
B. adusta 8258	Anthracene, 5 μg/ml	Bran flakes medium	95			5 d	Pickard et al., 1999
	Phenanthrene, 5 μg/ml		30				
	Pyrene, 5 μg/ml		35				
B. adusta 7308	Anthracene, 5 μg/ml		96				
	Phenanthrene, 5 μg/ml		40				
	Pyrene, 5 μg/ml		44				
P. ostreatus 7964	Anthracene, 5 μg/ml		92				
	Phenanthrene, 5 μg/ml		50				
	Pyrene, 5 μg/ml		35				
Coriolopsis gallica 8260	Anthracene, 5 μg/ml		90				
	Phenanthrene, 5 μg/ml		42				
Many white-rot fungi	Anthracene	Ligninolytic stationary cultures	60	Anthraquinone		21 d	Vyas et al., 1994
Bjerkandera sp. strain BOS55	Total PAHs, 50 mg/l in 2% acetone, 30 mg/l in 2% ethanol	Basal nutrient solution	53 / 80			7 d	Field et al., 1996a
Penicillium sp. and white-rot fungi	Anthracene, 10 mg/l	Kirk or Czapek–Dox medium	low	Unknown metabolites		60 d	Sack and Gunther, 1993
Laetiporus sulphureus	Phenanthrene, 50 mg/l		100	Unknown metabolites		35 d	
	Fluorene, 50 mg/l		100	Unknown metabolites		60 d	
	Fluoranthene, 50 mg/l		45	Unknown metabolites		60 d	
Trametes versicolor	Phenanthrene, 50 mg/l		100	Unknown metabolites		45 d	
	Fluorene, 50 mg/l		100	Unknown metabolites		60 d	
Penicillium sp. M1	Phenanthrene, 50 mg/l		100	Unknown metabolites		60 d	
	Fluoranthene, 50 mg/l		50	Unknown metabolites		60 d	
Oxysporus sp.	Anthracene, 0.01 g/l	GS liquid medium	94			2 d	Krivobok et al., 1998
Rhizoctonia solani			86				

Fungus	Substrate	Medium	%	Products	Time	Reference
Cladosporium herbarum						
Drechslera spicifera						
Verticillium lecanii			85			
R. solani	Anthracene, 0.84 mg/l	Liquid medium	79 / 77	*trans*-1,2-Dihydrodiol, three xyloside conjugates	6 d	Sutherland et al., 1992
C. elegans IM 1785/21 Gp	Anthracene, 250 mg/l; Phenanthrene, 250 mg/l	Liquid medium	99 / 82 / 99	9,10-Anthraquinone (22%)	7 d	Lisowska and Dlugonski, 1999
Irpex lacteus	Anthracene; Fluoranthene; Pyrene, each 25 ppm	MEG medium	60 / 15 / 40	Anthraquinone	2 w	Novotny et al., 2000
Trametes trogii	Anthracene, 250–500 ppm		>90		12–24 d	Levin et al., 2003
Aspergillus niger	Phenanthrene, 100 µl; Pyrene, 100 µl	Czapek–Dox medium		1- and 2-Phenanthrols, 1-methoxyphenanthrene; 1-Pyrenol, 1-methoxypyrene	63 d	Sack et al., 1997a
C. elegans	Phenanthrene, 37 kBq	Sabouraud broth	>99	Phenanthrene *trans*-3,4-dihydrodiol, 1-phenanthryl β-D-glucopyranoside, 2-hydroxy-1-phenanthryl β-D-glucopyranoside	8 d	Casillas et al., 1996
Syncephalastrum racemosum			55	Phenanthrene *trans*-3,4-dihydrodiol, phenanthrene *trans*-9,10-dihydrodiol, 2-, 3-, 4-, and 9-phenanthrols		
A. niger			36	Phenanthrene *trans*-9,10-dihydrodiol, 1, and 2-phenanthrols		
T. versicolor	Phenanthrene; Pyrene	N-limited liquid medium	14 CO_2 / 2.4 CO_2	Phenanthrene 9,10-dihydrodiol; Pyrene *trans*-4,5-dihydrodiol	63 d	Sack et al., 1997b

TABLE 8.1 Continued

Fungus	PAH Conc.	Medium	Degradation/ Mineralization Rate (%)	Metabolic Products	Pathway	Duration (hours/ days/ weeks)	Reference
Kuehneromyces mutabilis	Phenanthrene, 100μl		4 CO_2	Phenanthrene 9, 10-dihydrodiol			
P. ostreatus	Pyrene, 100μl	Basidiomycetes Rich medium	1.4 CO_2	Pyrene *trans*-4,5-dihydrodiol		11d	Bezalel et al., 1996b
	Phenanthrene, 2.5 mg nonlabeled and 1μCi labeled		94	Phenanthrene *trans*-9, 10-dihydrodiol (28%), 2,2′-diphenic acid (17%)	1		
Irpex lacteus	Phenanthrene	Liquid culture	8 CO_2	Polar metabolites		21d	Song, 1997
	Pyrene		10 CO_2				
Coriolus versicolor	Phenanthrene		10 CO_2				
	Pyrene		9 CO_2				
Phanerochaete chrysosporium	Phenanthrene	Non-N-limiting cultures	2 CO_2			25d	Barclay et al., 1995
	Benzo[a]pyrene, 76μg/l		3 CO_2				
Three species of *Cunninghamella*	Fluorene, 0.005 g/l	Synthetic liquid medium	94–98	9-Fluorenol, 9-fluorenone		2d	Garon et al., 2000
B. adusta	0.005 g/l		89	9-Fluorenol, 9-fluorenone			
Drechslera spicifera	0.005 g/l		93	9-Fluorenol, 9-fluorenone			
Absidia cylindrospora	Fluorene, 0.005 g/l	Liquid medium	98			2d	Garon et al., 2004
C. elegans ATCC 36112	2-Nitrofluorene		81	2-Nitro-9-fluorenol, 2-nitro-9-fluorenone, 6-hydroxy-2-nitrofluorene, sulfate conjugates		6d	Pothuluri et al., 1996a
Four species of *Cunninghamella*	Fluoranthene, 10 mg/l	Liquid synthetic medium	86–98			4d	Salicis et al., 1999
Cryptococcus albidus			93				

Organism	Substrate	Medium	%		Products	Time	Reference
C. elegans	Fluoranthene				Fluoranthene trans-2,3-dihydrodiol, 8- and 9-hydroxyfluoranthene trans-2,3-dihydrodiols, two glucoside conjugates	5 d	Pothuluri et al., 1990
C. elegans	Chrysene, 5mg	Sabouraud dextrose broth	45	1	Sulfate conjugates of 2,8-dihydroxychrysene, 2-hydroxychrysene	6 d	Pothuluri et al., 1995
Four filamentous fungi	Chrysene, 0.1mg/ml	MYPD medium	3		trans-1,2-Dihydroxy-1,2-dihydrochrysene	10d	Kiehlmann et al., 1996
Penicillium terrestre	Pyrene, 50mg/l 100mg/l	Basal salts medium	75 67			28d	Saraswathy and Hallberg, 2002
Penicillium janthinellum	Pyrene, 50mg/l 100mg/l		57 31				
Trichoderma harzianum	Pyrene 50mg/l 100mg/l		65 34				
Fusarium solani	Pyrene 40mg/l	Liquid mineral medium	32			17 d	Romero et al., 2002
Rhodotorula glutinis			37				
A. niger SK 9317	Pyrene, 20mg/l	20-L fermentor Mineral salts medium	>99	1	1-Hydroxypyrene, 1,6- and 1,8-pyrenequinones, 1,6- and 1,8-dihydroxypyrenes, 1-pyrenyl sulfate, 1-hydroxy-8-pyrenyl sulfate	200h	Wunder et al., 1994
Penicillium glabrum	Pyrene				1-Hydroxypyrene, 1,6- and 1,8-dihydroxypyrenes, 1,6- and 1,8-pyrenequinones, 1-pyrenyl sulfate, 1-methoxypyrene, 1,6-dimethoxypyrene		Wunder et al., 1997
P. janthinellum	Pyrene, 0.1mg/ml	MYPD medium	100		1-Pyrenol, 1,6- and 1,8-pyrenediols, 1,6- and 1,8-pyrenequinones	96h	Launen et al., 1995
	Benzo[a]pyrene, 0.1mg/ml				9-Hydroxybenzo[a]pyrene	7d	

TABLE 8.1 *Continued*

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Fungus	PAH Conc.	Medium	Degradation/ Mineralization Rate (%)	Metabolic Products	Pathway	Duration (hours/ days/ weeks)	Reference
Syncephalastrum racemosum	Pyrene, 0.1 mg/ml			1-Pyrenol, 1,6- and 1,8-pyrenediols, 1,6- and 1,8-pyrenequinones		96h	
	Benzo[a]pyrene, 0.1 mg/ml			9-Hydroxy-benzo[a]pyrene		7d	
P. janthinellum	Benzo[a]pyrene, 50 mg/l	MYPD medium	61	Water-soluble metabolites		56d	Stanley et al., 1999
Pleurotus eryngii	Benzo[a]pyrene, 100 μM	Liquid medium	75			15d	Rodriguez et al., 2004
P. pulmonarius			75			15d	
Crinipellis maxima	Pyrene, 20 mg/l	Modified mineral salts medium	82	1,6- and 1,8-Dihydroxypyrenes/1, 6- and 1,8-pyrenequinones, 1-pyrenyl sulfate		14d	Lange et al., 1996
Marasmius rotula			96	1,6- and 1,8-Dihydroxypyrenes/1, 6- and 1,8-pyrenequinones, 1-hydroxypyrene			
Marasmiellus ramealis			51	1-Hydroxypyrene, 1-pyrenyl sulfate, trans-4, 5-dihydro-4, 5-dihydroxypyrene	1		
C. elegans	Benz[a]anthracene, 23 nM	Sabouraud dextrose broth		trans-3,4-, trans-8,9-, trans-10,11-Dihydrodiols, benz[a]anthracene tetraol	1	2d	Cerniglia et al., 1994
Marasmiellus troyanus	Benzo[a]pyrene, 20 μg/ml	Sabouraud dextrose broth (Mycelia)	95			17d	Wunch et al., 1997
Aspergillus ochraceus			45	Detectable metabolites			
Hericium erinaceus		Extracellular filtrate	45				

Organism	Substrate	Medium	%	Products	Time	Reference
A. ochraceus	Benzo[a]pyrene, 0.4 mM	Liquid medium		trans-9,10-Dihydroxy-9,10-dihydrobenzo[a]pyrene, trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene, trans-4,5-dihydroxy-4,5-dihydrobenzo[a]pyrene, benzo[a]pyrene 1,6- and 3,6-quinones, 3- and 9-hydroxybenzo[a]pyrenes	2 d	Datta and Samanta, 1988
Fusarium solani	[7,10-^{14}C]Benzo[a]pyrene, 302 mg with 3 µCi	Mineral salts medium	1.2 CO_2	1,6- and 3,6-Benzo[a]pyrene quinones	256h	Veignie et al., 2002
F. solani	Benzo[a]pyrene 5×10^{-4} M/l	MM medium	6.8		15 d	Veignie et al., 2004
F. solani *Trichoderma viride* *Fusarium oxysporum*	Benzo[a]pyrene, 0.4 mM	Mineral medium	58 50 30		30d	Verdin et al., 2004
Cladosporium sphaerospermum	Benzo[a]pyrene	Liquid medium	18		4 d	Potin et al., 2004b
Cyclothyrium sp.	Anthracene, 2mg	Sabouraud dextrose broth		trans-1,2-Dihydroxy-1,2-dihydroanthracene	192h	da Silva et al., 2003, 2004
	Phenanthrene, 2mg		90	trans-3,4-Dihydroxy-3,4-dihydrophenanthrene, trans-9,10-Dihydroxy-9,10-dihydrophenanthrene, 2-hydroxy-7-methoxy-phenanthrene, 1-, 3-, and 4-hydroxyphenanthrenes	96h	
	Pyrene, 2mg			trans-4,5-Dihydroxy-4,5-dihydropyrene, pyrene-1,6-and pyrene-1,8-quinones, 1-hydroxypyrene	96h	
Penicillium ochrochloron strain LP	Pyrene, 50mg/l	Basal salts medium	75		28d	Saraswathy Hallberg, 2005

8.4.1   Fungal Metabolism of Naphthalene

Naphthalene is a dicyclic aromatic hydrocarbon whose oxidation is demonstrated by several species of fungi. Several fungi metabolize naphthalene to naphthalene *trans*-1,2-dihydrodiol, 1- and 2-naphthols, 4-hydroxy-1-tetralone, and glucuronide and sulfate conjugates (Cerniglia et al., 1978; Hofmann, 1986). Metabolic pathways for the degradation of naphthalene by fungi have also been depicted (Cerniglia and Gibson, 1977). Less toxicity of conjugation products than of the parent PAH suggests the applicability of *C. elegans* to remediate naphthalene-contaminated soils. The yeast *Candida lipolytica* transforms naphthalene to 1- and 2-naphthols, 4-hydroxy-1-tetralone, and *trans*-1,2-dihydroxy-1,2-dihydronaphthalene, and a pathway has been proposed (Cerniglia and Crow, 1981).

8.4.2   Fungal Metabolism of Acenaphthene

Acenaphthene is a tricyclic fused aromatic hydrocarbon and is considered nonmutagenic. Acenaphthene has been found to induce cytological and nuclear changes in plants and microorganisms (USEPA, 1987). Little is known about the metabolism of acenaphthene by microorganisms. *C. elegans* ATCC 36112 metabolized 64% of [1,8-^{14}C]acenaphthene on Sabouraud dextrose medium, producing seven metabolites, in work of Pothuluri et al. (1992b).

8.4.3   Fungal Metabolism of Anthracene

Anthracene is a noncarcinogenic tricyclic aromatic hydrocarbon found frequently in PAH-contaminated sediments. *Bjerkandera adusta* converts less than 15% anthracene to anthraquinone (Schutzendubel et al., 1999). *Pleurotus ostreatus* exhibits a good correlation between the elimination of anthracene and the accumulation of anthraquinone. *Coriolopsis polyzona*, *P. ostreatus*, and *Trametes versicolor* can further degrade anthraquinone, and its degradation does not appear to be a rate-limiting step (Vyas et al., 1994). Anthracene is metabolized efficiently to a dead-end metabolite, anthraquinone, by strains of the genera *Bjerkandera*, *Phanerochaete*, *Trametes*, *Ramaria*, and Agaricales (Field et al., 1992) and *Rhizoctonia solani* (Sutherland et al., 1992). *Bjerkandera* sp. strain BOS55 degraded 16 EPA PAHs from polluted soil extracted with either 2% acetone or ethanol (Field et al., 1996a). Of 39 strains of Micromycetes, 19 strains degraded 50% or more of anthracene (Krivobok et al., 1998). Zygomycetes is the most efficient group, with a mean degradation of 81%; Melanconiales are least efficient, with a 41% mean degradation. Among 19 effective strains, nine are new to the literature. Of nine fungal strains, *Cunninghamella elegans* IM 1785/21 Gp was the best performer (Lisowska and Dlugonski, 1999). A pathway proposed for the metabolism of anthracene by *P. ostreatus* is shown in Figure 8.2 (Bezalel et al., 1996c).
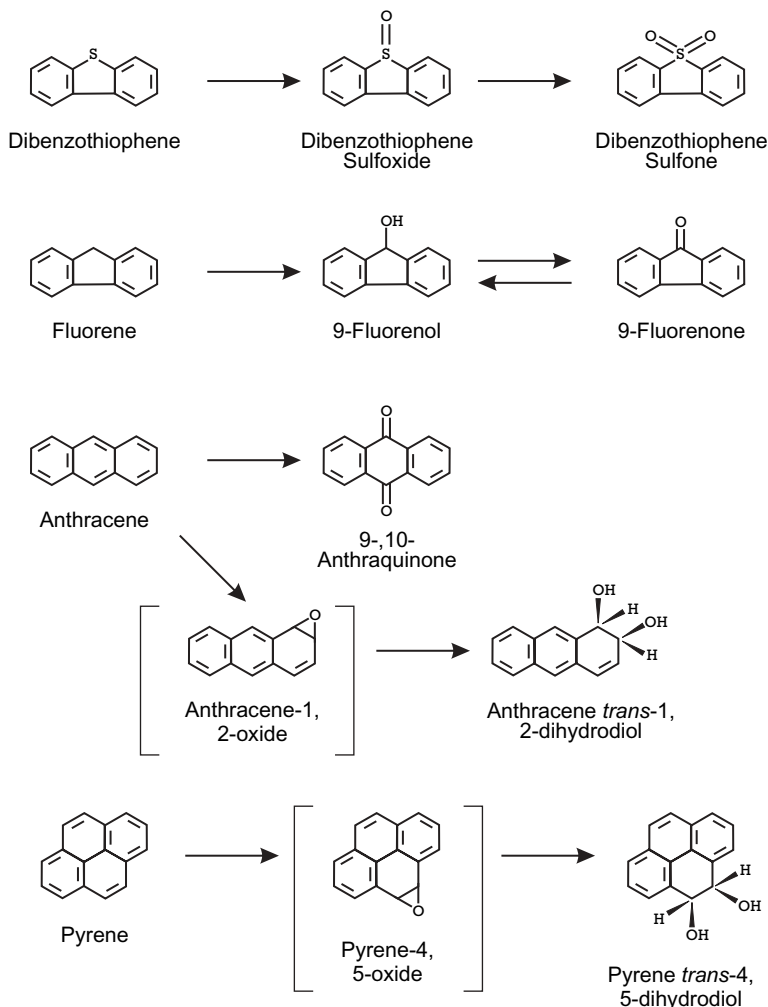
Figure 8.2 Tentative pathways for the metabolism of pyrene, anthracene, fluorene, and dibenzothiophene by *Pleurotus ostreatus*. [Reprinted from Bezalel et al. (1996c), copyright © with permission from the American Society for Microbiology and the authors.]

8.4.4 Fungal Metabolism of Phenanthrene

Phenanthrene is a noncarcinogenic and nonmutagenic tricyclic aromatic hydrocarbon found in aquatic and terrestrial oil-contaminated sediments. Of 20 fungal isolates, *Pleurotus ostreatus* UAMH 7964 performed best on 2% bran flakes medium containing phenanthrene (Pickard et al., 1999). *Trichosporon penicillatum* showed the highest capacity for phenanthrene biotransformation (MacGillivray and Shiaris, 1993). Phenanthrene 9,10-dihydrodiol

is a major metabolite produced by *Trametes versicolor*, *Kuehneromyces muta-bilis*, *Agrocybe aegerita*, and *Flammulina velutipes* (Sack et al., 1997b). White-rot fungi transform phenanthrene in the C9,10 positions (K-region); all other fungi produce metabolites substituted in the C1,2, C3,4, and C9,10 positions, similar to soil fungi. The pattern of mineralization by *Phanero-chaete chrysosporium* INA-12 did not correlate with the formation of LiP activity (Barclay et al., 1995). The sorption of radiolabeled phenanthrene is 40%. Figure 8.3 shows the phase I and phase II metabolism of phenanthrene by three fungi (Casillas et al., 1996). Pathways for the oxidation of phenan-threne by different species of fungi have also been proposed (Cerniglia et al., 1992; Sutherland et al., 1995; Bezalel et al., 1996b; da Silva et al., 2004).

8.4.5   Fungal Metabolism of Fluorene

Fluorene, a tricyclic PAH with a five-membered ring, is noncarcinogenic but highly toxic to fish and aquatic algae. Fluorene was shown to contain 9.5% carcinogenic PAHs in a study on the bioremediation of a contaminated soil using phytotoxicity tests (Baud-Grasset et al., 1993). Fluorene is present in most PAH mixtures, and its structure has been found in several mutagenic and/or carcinogenic PAHs, such as 2-aminofluorene, 2-nitrofluorene, and 2-acetylaminofluorene. Fluorene has been used as a model compound for study-ing the biodegradation of PAHs.

  Less is known on the fungal metabolism of fluorene. Of 30 strains of Micromycetes, 12 strains were the best degraders (Garon et al., 2000). Three strains of *Cunninghamella* were highly efficient, with a mean degradation rate of 96%. Two strains each from Ascomycetes and Basidiomycetes and three from Deuteromycetes were also highly efficient. Eleven strains were new to the literature. Oxidation of aliphatic ring in fluorene resulted in the formation of two monooxygenated metabolites, 9-fluorenol and 9-fluorenone, by most of the strains. *Agaricus bitorquis*, *Aspergillus terreus*, *Penicillium italicum*, and *Oxysporus* sp. revealed the formation of a major metabolite, 9-fluorenol (>56%), whereas *Cylindrocarpon destructans* and *Dichotomomyces cejpii* produced 9-fluorenol and 9-fluorenone as the major metabolites. The forma-tion of monooxygenated products can be a first step in the detoxification process. *Pleurotus ostreatus* metabolized 96% of fluorene in Basidiomycetes rich medium (BRM) with the formation of 9-fluorenol and 9-fluorenone, and a pathway has also been proposed (Bezalel et al., 1996c). However, the rate of fluorene mineralization by *P. ostreatus* is very low (Bezalel et al., 1996a). *C. elegans* detoxifies fluorene with the formation of 9-fluorenol, 9-fluorenone, and 2-hydroxy-9-fluorenone (Pothuluri et al., 1993). *P. ostreatus* exhibits a good correlation between the elimination of fluorene and the accumulation of 9-fluorenone (Schutzendubel et al., 1999). *C. elegans* metabolizes about 81% of [9-^{14}C]-2-nitrofluorene, forming six metabolites after 144 hours of incubation (Pothuluri et al., 1996a).
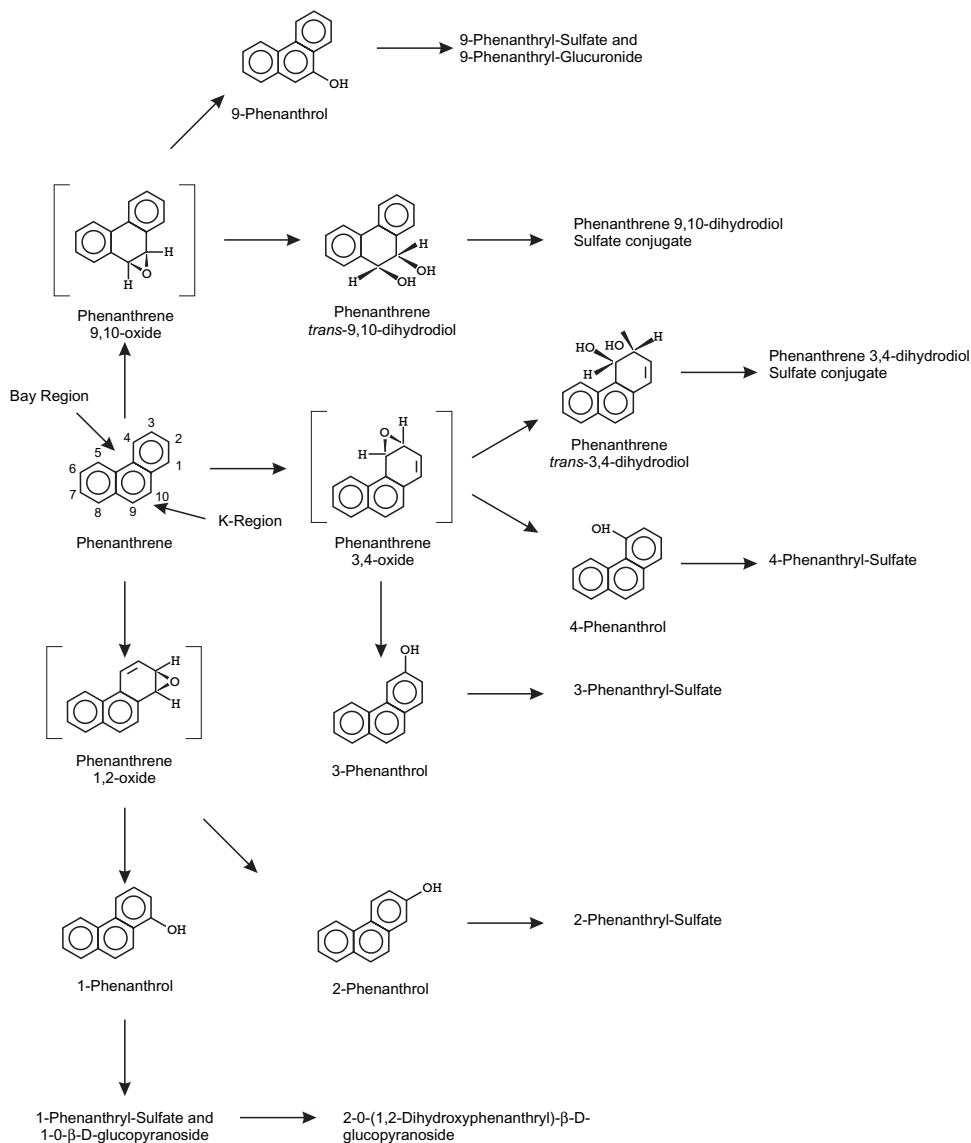
Figure 8.3 Tentative pathways for the phase I and phase II metabolism of phenanthrene by *Aspergillus niger* ATCC 6275, *Cunninghamella elegans* ATCC 9245, and *Syncephalastrum racemosum* UT-70. [Reprinted from Casillas et al. (1996), copyright © with permission from Springer Science and Business Media and the authors.]

Surfactants can improve the rate and extent of biodegradation by fungi of fluorene in pure cultures. Solubilization of fluorene is enhanced significantly in the presence of three surfactants on the efficiency scale Tween 80 > Triton X-100 > sodium dodecyl sulfate (Garon et al., 2002). Biodegradation of flourene was enhanced by *Doratomyces stemonitis* (46 to 62%) and *Penicillium chrysogenum* (28 to 61%) in the presence of Tween 80 after 2 days. The efficiency of Tween 80 can be attributed to the structure of micelles and/or to the mechanism of interaction between micellized surfactant and fungi. The degradation of fluorene by *Dichotomomyces cejpii* is inhibited by Triton X-100.

8.4.6   Fungal Metabolism of Fluoranthene

Fluoranthene is consistently the most abundant tetracyclic aromatic hydrocarbon in environmental samples. It has been recognized to be cytotoxic, mutagenic, and potentially carcinogenic. Due to its abundance, it is considered more of a potential health hazard than the widely studied but less abundant carcinogen benzo[*a*]pyrene. Current knowledge on the fungal metabolism of fluoranthene is limited, and the degradation pathways are unknown.

Fluoranthene is better degraded in cometabolism than as a sole source of carbon (Cerniglia, 1993). Of 39 strains of Micromycetes, 16 strains degraded 60% or more fluoranthene (10 mg/l) after 4 days (Salicis et al., 1999). Zygomycetes of the genus *Cunninghamella* appeared to be the most efficient group, with a degradation range of 86 to 98% in liquid synthetic medium. Four strains of Deuteromycetes and three strains of Basidiomycetes showed good degradation capacities. Ten good performant strains were new to the literature. A low mean adsorption (4%) onto fungi has been determined for the strains that show good fluoranthene degradation. Some strains of *Colletotrichum dematicum* and *Penicillium italicum* adsorb 47% and 43% of fluoranthene, respectively. *Irpex lacteus* removed 15% of fluoranthene on MEG medium after 2 weeks (Novotny et al., 2000). An HPLC elution profile identified five metabolites of fluoranthene after 120 hours by *C. elegans* (Pothuluri et al., 1990).

8.4.7   Fungal Metabolism of Chrysene

Chrysene is a tetracyclic aromatic hydrocarbon, a weak carcinogen, and has been shown to be genotoxic through use of a mutagenicity assay technique. Chrysene has been metabolized to form non-K-region dihydrodiol and diol epoxides (Sims and Grover, 1981). Pothuluri et al. (1995) were the first to report on the transformation of chrysene by *C. elegans*. Two major metabolites, sulfate conjugates of 2,8-dihydroxychrysene and 2-hydroxychrysene, have been identified by UV and ^{1}H-NMR and account for 33% of the total metabolism. A proposed pathway for the degradation of chrysene by *C. elegans* is shown in Figure 8.4. Four filamentous fungi (i.e., *Penicillium*
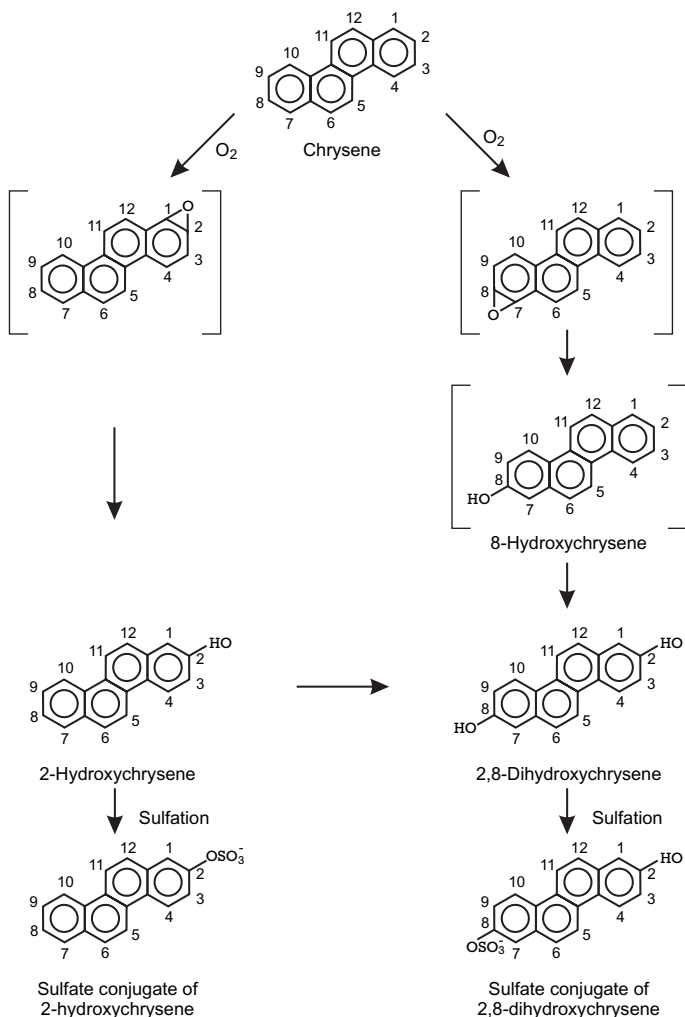
Figure 8.4 Tentative pathways for the metabolism of chrysene by *Cunninghamella elegans*. Structures in parentheses are proposed intermediates but not detected. [Reprinted from Pothuluri et al. (1995), copyright © with permission from the National Research Council, Canada.]

janthinellum, *Syncephalastrum racemosum*, and two species of *Penicillium*) metabolized chrysene and Tween 80 after 10 days at 24ºC (Kiehlmann et al., 1996). About 3% of chrysene was converted to one of the metabolites, *trans*-1,2-dihydroxy-1,2-dihydrochrysene in 6 days. Other metabolites have not been structurally identified. Metabolites constituted 90% of the metabolism.

8.4.8   Fungal Metabolism of Pyrene

Pyrene is a fused tetracyclic aromatic hydrocarbon employed as an indicator for monitoring PAH-contaminated wastes. Pyrene is not genotoxic, but its quinone intermediates are mutagenic and more toxic than the parent compound. It has been used as a model compound to measure binding to DNA and to examine the photochemical and biodegradation of other PAHs.

Based on the dry weights and oxidation of pyrene in both 50 and 100 mg/l, *Penicillium* strains rank in the order *P. terrestre* > *P. simplicissimum* > *P. funiculosum* > *P. janthinellum* (Saraswathy and Hallberg, 2002). This appears to be the first report of the utilization by fungi of pyrene as a sole carbon and energy source. *Fusarium solani* and the yeast *Rhodotorula glutinis* utilize pyrene as the sole source of carbon in liquid mineral medium (Romero et al., 2002). A higher ability to transform pyrene with $^{14}CO_2$ evolution has been found in *Rhodotorula minuta* and *R. glutinis*, with the consumption of 35% at an initial concentration of 40 mg/l. Of 41 isolates of Micromycetes, 10 strains were found to metabolize pyrene highly (>2.4 mg/g dry weight) (Ravelet et al., 2000). The taxonomic distribution of high degraders comprises two from Zygomycetes, six from Deuteromycetes, and one each from Dematiaceae and Sphaeropsidales. Zygomycetes is found to be one of the most efficient taxonomic groups, especially *Mucor racemosus* var. *sphaerosporus*, which degrades pyrene highly (3.26 mg/g dry weight). Among 10 good performers, nine are new to the literature and show good potential for mycoremediation. Addition of pyrene produced mycelial pellets in two strains of *Penicillium ochrochloron* (Saraswathy and Hallberg, 2005).

Pyrene degradation by *Penicillium glabrum* TW 9424 in submerged cultures led to the identification of several metabolites (Wunder et al., 1997). This is the first report on the isolation of methoxylated metabolites of PAHs from fungal cultures. *I. lacteus* removed nearly 40% pyrene after 2 weeks (Novotny et al., 2000). Circular dichroism (CD) spectra reveal that pyrene *trans*-4,5-dihydrodiol contains 63% 4R,5R enantiomer and 37% 4S,5S enantiomer with an optical purity of 26% (Bezalel et al., 1996c). Pyrene metabolism occurs at the 4,5 bond (K-region), resulting in an epoxide that hydrates to produce pyrene *trans*-4,5-dihydrodiol.

Pyrene-oxidizing strains belong to genera and species of Zygomycetes, Ascomycetes, and Deuteromycetes but not Basidiomycetes (Launen et al., 1995). *Penicillium* spp. of the subgenus *Furcatum* are the most common in highly contaminated soils. In *Penicillium janthinellum* SFU403, five metabolites account for 20% of the metabolism (i.e., 14% [^{14}C]pyrene oxidized and 25% cell-associated). Almost all pyrene is utilized within 72 to 96 hours. Response-surface methodology has been employed to determine the optimum growth conditions for pyrene oxidation by *P. janthinellum* SFU403 (Launen et al., 1999). The total optimized biotransformation to oxidation products is approximately 100%. Launen et al. (2000) also showed that pyrenequinones (PQs) bind irreversibly to cells in *P. janthinellum* SFU403. About 40% of the

inextractable products are bound residues derived from pyrene metabolites, and the balance (60%) is attributed to strong sorption of unreacted pyrene. Oxygen consumption and electron paramagnetic resonance spectroscopy clearly demonstrate that the intracellular reductants nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione (GSH) can reduce PQs by one-electron oxidation directly to the corresponding semiquinone anion radicals in vitro. Similar reduction of naphthoquinone and benzo[*a*]pyrene-7,8-dione to the corresponding semiquinone radicals by the NADPH has also been demonstrated by Flowers et al. (1997), and the semiquinone radicals can bind to DNA and redox cycle with oxygen. This supports the fact that the PQs are metabolized to bound residues, possibly via semiquinone intermediates. No studies have been performed to confirm in vivo metabolism. Pyrene metabolism by *P. janthinellum* is shown in Figure 8.5.

Pyrene metabolism by the basidiomycete *Crinipellis stipitaria* JK375 leads to the identification of PQs and hydroxylated derivatives (Lambert et al., 1994). *C. stipitaria* JK364 transforms pyrene to *trans*-4,5-dihydro-4,5-dihydroxypyrene, 1-hydroxypyrene, and 1-pyrenyl sulfate (Lange et al., 1994). This strain has also been found to mineralize [^{14}C]pyrene to $^{14}CO_2$ and to degrade it to [^{14}C]acetic acid. Pyrene is also oxidized by different species and strains of basidiomycete genera of *Crinipellis*, *Marasmius*, and *Marasmiellus*
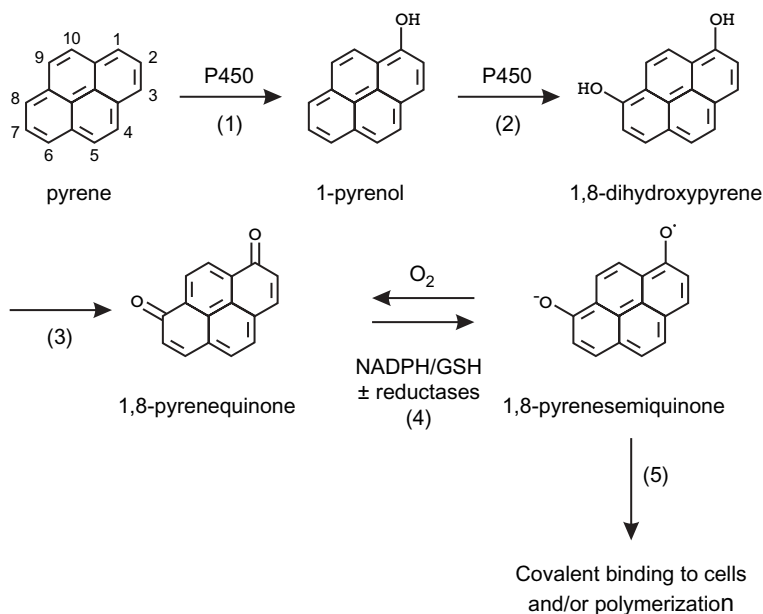
Figure 8.5 Pyrene metabolism by *Penicillium janthinellum* SFU403. [Reprinted from Launen et al. (2000), copyright © with permission from Springer Science and Business Media and the authors.]
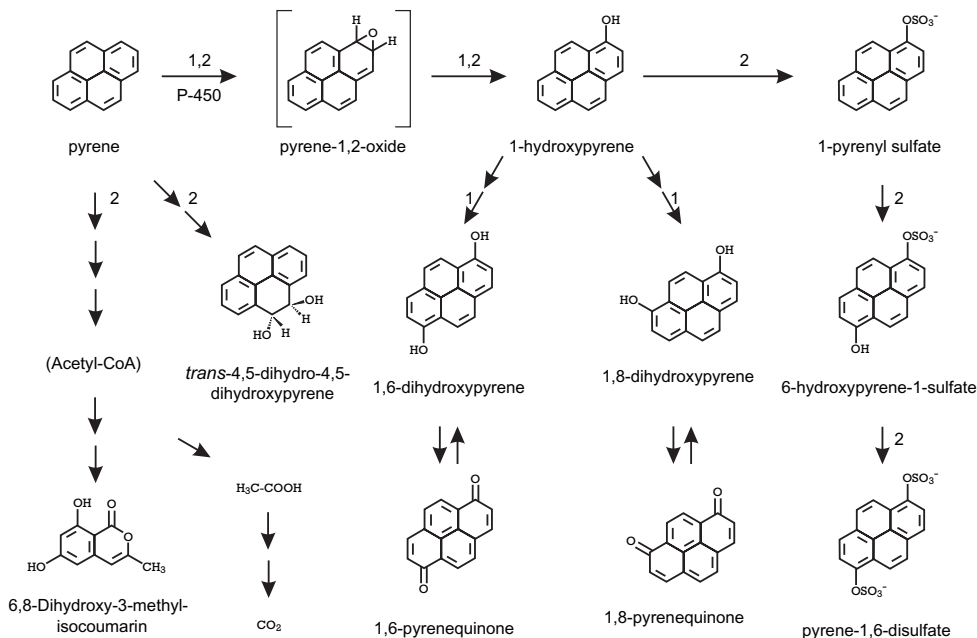
Figure 8.6   Tentative pathways for the metabolism of pyrene by *Crinipellis stipitaria* JK375 (1) and JK364 (2). [Reprinted from Lange et al. (1996), copyright © with permission from the National Research Council, Canada.]

(Lange et al., 1996). All strains showed the ability to metabolize pyrene in submerged culture, but most of the strains transform pyrene more effectively in complex medium. More than 60% pyrene and metabolites, including water-soluble sulfates and disulfites, account from the mycelia in fungal cultures. A specific pattern of metabolites was formed in each strain, which depends on the culture medium. The major pathways of pyrene metabolism by *C. stipitaria* are shown in Figure 8.6. Pathways for the metabolism of pyrene by *A. niger* SK 9317 (Wunder et al., 1994), *P. ostreatus* (Bezalel et al., 1996c), and *Cyclothyrium* sp. (da Silva et al., 2004) were also proposed.

8.4.9   Fungal Metabolism of Benz[*a*]anthracene

Benz[*a*]anthracene is a widely distributed tetracyclic aromatic hydrocarbon but is a weaker carcinogen than benzo[*a*]pyrene. Limited information is available on the metabolism of benz[*a*]anthracene by microorganisms. Two yeasts, *Candida krusei* and *Rhodotorula minuta*, are able to degrade benz[*a*]anthracene, but the degradation is very limited (MacGillivray and Shiaris, 1993). *C. elegans* metabolizes [^{14}C]benz[*a*]anthracene to metabolites such as *trans*-3,4- (4%), *trans*-8,9- (90%), *trans*-10,11- (6%), and dihy-

drodiols grown on Sabouraud dextrose broth (Cerniglia et al., 1994). Benz[*a*]anthracene tetraol, 8β,9α,10α,11β-tetrahydroxy-8α,9β,10β,11α-tetrahydro-benz[*a*]anthracene, was identified as a metabolite, and this may be considered as an additional oxidation product of either benz[*a*]anthracene 8,9- or 10,11-dihydrodiol. This was a first report on the formation of biologically produced benz[*a*]anthracene tetraol. A pathway was also proposed for the initial metabolism of benz[*a*]anthracene by *C. elegans*.

8.4.10   Fungal Metabolism of Benzo[*a*]pyrene

Benzo[*a*]pyrene is a fused pentacyclic aromatic hydrocarbon and is one of the most potent carcinogens. Of 17 strains of filamentous fungi, the mycelia of six species eliminated more than 40% of benzo[*a*]pyrene at 25°C in 17 days (Wunch et al., 1997). A rare fungus, *Marasmiellus troyanus*, has the capacity to achieve extremely high removal rates: up to 95%. *Aspergillus ochraceus* was the only fungus to show detectable metabolites in cultures. Extracellular filtrate of *Hericium erinaceus* revealed the highest removal rate: 45%. The sorption of radiolabeled benzo[*a*]pyrene by *P. chrysosporium* INA-12 is 22% (Barclay et al., 1995). *Candida lipolytica* shows the detection of 3- and 9-hydroxybenzo[*a*]pyrenes within 2 days at an initial concentration of 200 mg/l in the presence of glucose (Cerniglia and Crow, 1981). Several metabolites have been detected from benzo[*a*]pyrene by *C. elegans* on Sabouraud dextrose broth (Cerniglia and Gibson, 1980a,b; Cerniglia et al., 1992). A pathway for the fungal oxidation of benzo[*a*]pyrene has also been proposed (Cerniglia et al., 1992). The sulfate and glucoside conjugates, 3-hydroxybenzo[*e*]pyrene and 3,10-dihydroxybenzo[*e*]pyrene formed during benzo[*e*]pyrene metabolism by *C. elegans* (Pothuluri et al., 1996b).

   The relative degradation per unit biomass for three mitosporic fungi, *Trichoderma viride*, *Fusarium solani*, and *Fusarium oxysporum*, is 39%, 17%, and 8%, respectively (Verdin et al., 2004). The laccase activities of *F. oxysporum*, a poor benzo[*a*]pyrene degrader, are five-times higher than those of *F. solani*. In *F. solani*, sodium azide, a laccase inhibitor, does not reduce benzo[*a*]pyrene degradation, but the laccase activity is diminished by 50%. This suggests that laccase is not involved in benzo[*a*]pyrene degradation. *T. viride* is able to degrade 50% of benzo[*a*]pyrene in the absence of laccase and peroxidase activities, suggesting that a different pathway is involved in PAH metabolism. *F. solani* can store benzo[*a*]pyrene in numerous intracellular vesicles, independent of the degradation rate (Verdin et al., 2005). About 5.3% of benzo[*a*]pyrene is incorporated in the biomass of *F. solani* (Veignie et al., 2002). *F. solani* oxidizes benzo[*a*]pyrene in the C1, C3, and C6 positions. Two metabolites, 1,6- and 3,6-benzo[*a*]pyrene quinones, have been identified, suggesting the involvement of cytochrome P450. Poor degradation (6.8%) occurred on benzo[*a*]pyrene in MM medium with *F. solani* after 15 days (Veignie et al., 2004). Potassium cyanide and piperonyl butoxide (both P450 and peroxidases inhibitors) enhanced benzo[*a*]pyrene degradation

significantly: to 16.3% and 22.6%, respectively. It appears that the increased availability of H_2O_2 in fungal cultures plays an important role in benzo[a]pyrene degradation. An alternative metabolic pathway based on free radical production, such as the reactive oxygen species (ROS) and hydroxyl radicals (·OH), which may be agents for the initiation of benzo[a]pyrene oxidation, was hypothesized.

8.5   MUTAGENICITY OF FUNGAL METABOLITES OF PAHs

In general, most of the fungal metabolites produced from PAH metabolism are less toxic than the parent compounds to other organisms, and this is an encouraging result of the detoxification process. However, oxidation products such as the quinones or hydroxy derivatives are often more toxic and are mutagenic.

Some minor metabolites produced as a result of substituted and methyl-substituted PAH metabolism by fungi are more mutagenic or carcinogenic than the parent compounds or major metabolites. These compounds are benz[a]anthracene *trans*-3,4-dihydrodiol (Cerniglia et al., 1980b), benzo[a]pyrene *trans*-7,8-dihydrodiol 9,10-epoxide (Cerniglia and Gibson, 1980b), 7,12-dimethylbenz[a]anthracene *trans*-3,4-dihydrodiol (Wong et al., 1983; McMillan et al., 1988), and 1-hydroxy-3-methyl-cholanthrene *trans*-9,10-dihydrodiol (Cerniglia et al., 1982). In *C. elegans*, Pothuluri et al. (1992a) showed a decrease in the mutagenic activity of five metabolites of fluoranthene toward *Salmonella typhimurium* with time, but was not eliminated. Similar reductions in the mutagenic activities of benz[a]anthracene, 7,12-dimethylbenz[a]anthracene, benzo[a]pyrene, or 3-methylcholanthrene have been shown toward *Salmonella typhimurium* reversion assay without total elimination (Cerniglia et al., 1985). The mutagenicity of 7,12-dimethylbenz[a]anthracene is reduced in cultures of *Syncephalastrum racemosum* after its biotransformation to glucuronide and sulfate conjugates (McMillan et al., 1988). *Crinipellis stipitaria* produces 1-hydroxypyrene, an initial metabolite in pyrene metabolism that shows cytotoxic and mutagenic properties (Lambert et al., 1995).

Sulfate, glucuronide, and glucoside conjugates produced as a result of PAH metabolism by fungi exhibit nonmutagenic properties. However, certain microorganisms contain hydrolytic enzymes that can eliminate the conjugative groups in the environment, thus restoring toxicity.

8.6   FUNGAL BIOREACTORS FOR REMOVAL OF PAHs

Bacterial bioreactors in use are the slurry phase, drum, and/or airlift type. A mixed bacterial culture is used for the biological conversion of anthracene-contaminated soil in rotating-drum bioreactors (Gray et al., 1994; Banerjee

et al., 1995), which include a combination of effective mixing with a high solids content and high aeration rates. Continuous-tank-stirred reactors (CSTRs) are also employed for the aerobic biodegradation of PAHs. Guieysse et al. (2001) obtained fast and complete degradation of PAHs and prevented their hazardous disappearance in a biphasic bioreactor. During the last decade, fungal bioreactors for the degradation of PAHs have been developed. These bioreactors are still in the developmental phase. Fungal bioreactors related to the degradation of PAHs are listed in Table 8.2.

8.6.1 Immobilized Bioreactors

The immobilized bioreactors are the best established for the removal of PAHs. *Phanerochaete chrysosporium* removes naphthalene, fluoranthene, and benzo[*a*]pyrene in cell-free or immobilized cultures, but 3 days are required for the acclimation of these PAHs (Liao et al., 1997). The strain ATCC 24725 was more resistant to PAH toxicity and removed 41% of benzo[*a*]pyrene within 30 days. Cell-free strains ATCC 24724 and ATCC 34541 producing ligninolytic peroxidases exhibited higher removal efficiencies of benzo[*a*]pyrene than strain ATCC 32629. Good removal of naphthalene was attributed to the maintenance of *Phanerochaete chrysosporium* activity and the ability to produce ligninolytic enzymes in a PAH-acclimated culture (Liao and Tseng, 1996). One metabolite was identified as catechol. Immobilized *P. chrysosporium* has been employed to eliminate surfactant Tween 80–solubilized PAHs in a rotating biological contactor (RBC) (Zheng and Obbard, 2002b). A schematic diagram of a bench-scale RBC reactor is shown in Figure 8.7. A reactor in the continuous mode effectively removed all PAHs for a specific loading rate and concentration for each PAH. A linear relationship exists between the inverse of PAH removal rate and the inverse of PAH loading rate. The PAH removal exceeded 90% after 60 hours, although a low level of MnP activity was detected. The RBC reactor can be operated for a period of 40 days without diminished effective removal capacity. Phenanthrene and pyrene were removed by a combination of fungal oxidation and disk foam adsorption, and benzo[*a*]pyrene was removed by fungal oxidation alone.

8.6.2 Closed-Batch Feed Bioreactors

Based on the principle of washing and bioremediation of soil, the Gebruder Huber system has been developed (Schuster and Blank-Huber, 1994; Reinert et al., 1996). This system employs an efficient combination of mechanical washing with biological degradation. An attempt is made to return decontaminated soils to the original site or to use them as an additive in construction material. Kamely et al. (1990) discussed cleanup strategies, including various reactor systems that employ a combination of soil extraction and biological treatment, similar to the Gebruder Huber system. These systems

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TABLE 8.2 Fungal Bioreactors for Removal of Polycyclic Aromatic Hydrocarbons

Fungus	Bioreactor Type	Bioreactor Volume	PAH Conc.	Removal/ Mineralization Rate (%)	Enzyme Activity (U/ml)	Duration (hours/days)	Reference
Phanerochaete chrysosporium ATCC 24725	Alginate-PAC immobilized/ aerated batch bioreactor	500 ml	Benzo[a]pyrene, 500 µg/l	41		30d	Liao et al., 1997
P. chrysosporium ATCC 24725	Aerated batch bioreactor	500 ml	Naphthalene, 10 mg/l	>95		7d	Liao and Tseng, 1996
P. chrysosporium	Immobilized in a RBC reactor/ batch operation	26 cm length 15 cm inner diameter	Synthetic PAH solution	90	MnP, low level	60h	Zheng and Obbard, 2002b
P. chrysosporium	Closed-batch feed bioreactor	500 ml, each fungal culture and soil-extracting reactors	Total PAHs, 41 g/kg Benzo[a]pyrene, 3 mg/kg	45 5 2.5 CO_2		36d	May et al., 1997
P. chrysosporium	Compost bioreactor	125-ml Erlenmeyer flasks	Benzo[a]pyrene	45		100d	McFarland et al., 1992
P. chrysosporium	Compost bioreactor	125-ml Erlenmeyer flasks	Benzo[a]pyrene	63		95d	McFarland and Qiu, 1995
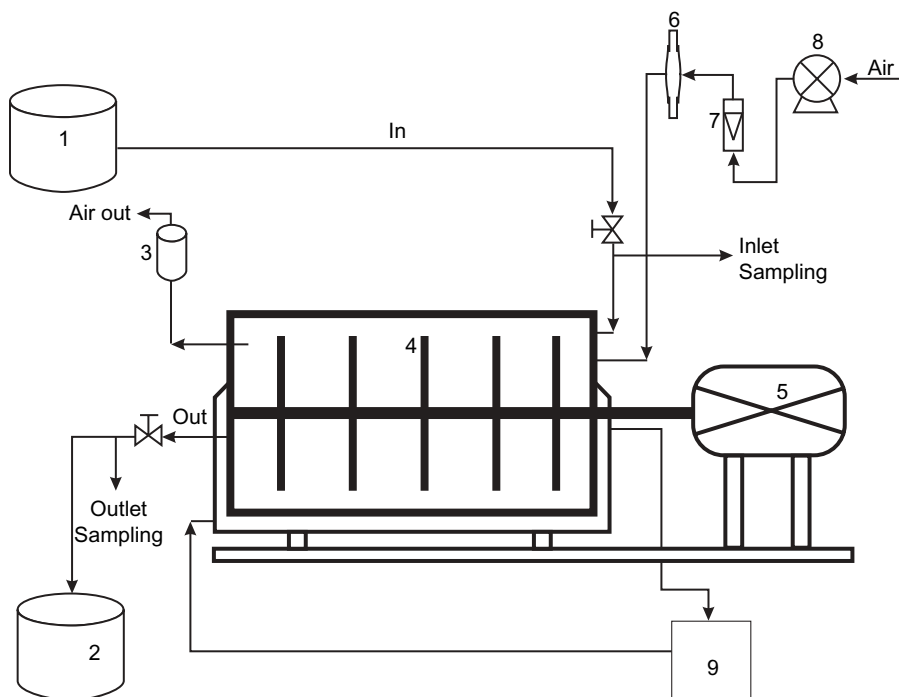
Figure 8.7 RBC containing immobilized *Phanerochaete chrysosporium*. 1, Feed PAH-contained wastewater tank; 2, effluent wastewater tank; 3, air condenser; 4, RBC reactor; 5, electrical motor; 6, air filter; 7, airflow meter; 8, air pump; 9, recycling water bath. [Reprinted from Zheng and Obbard (2002b), copyright © with permission from Elsevier.]

allow enhanced bioavailability of PAHs by fungi. In Germany, many companies have developed soil purification plants.

A model reactor system has been constructed of separate soil extraction and incubation systems with *Phanerochaete chrysosporium* at 39ºC (May et al., 1997). Construction of a reactor system is shown in Figure 8.8. The system is a closed batch feed design equipped with gas scrubbers, thus preventing toxic metabolites escape and stripping. The system allows simultaneous determination of mineralization and mass recovery. The rate of degradation of lower PAHs was 70 to 100% and of higher PAHs was 6 to 50%. HPLC analyses reveal high levels of polar ^{14}C-labeled metabolites. This degradation is due to direct polymerization on the soil matrix. The fungus transforms PAHs to quinones that can polymerize rapidly.
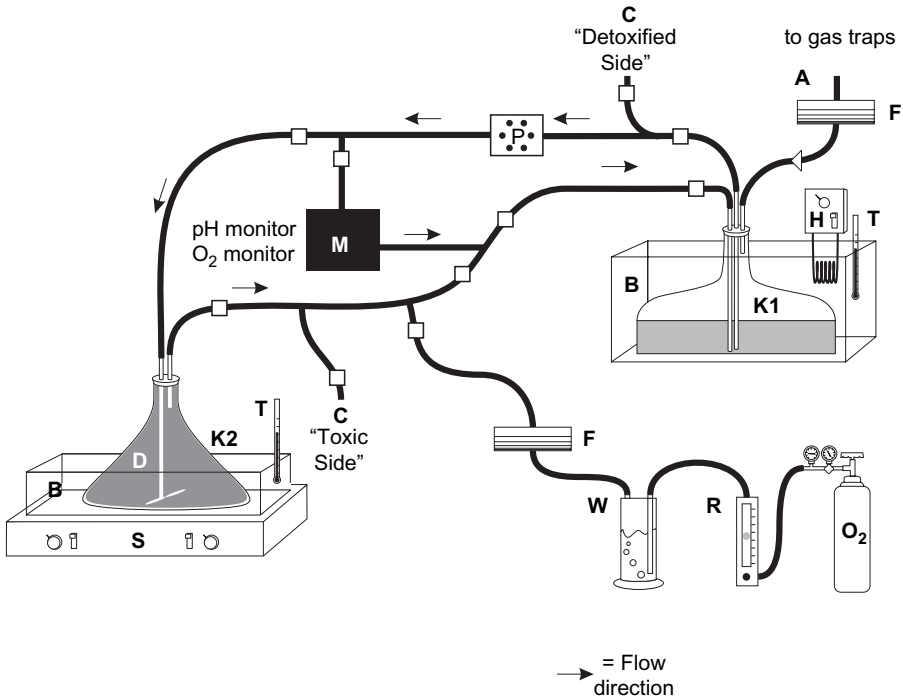
Figure 8.8   Closed-batch feed reactor system with *Phanerochaete chrysosporium*. Open square, clamp; A, pressure exit; B, heating bath; C, medium sampling exit; D, contaminated soil; F, 0.2-μm sterile filter; H, bath heater; K1, fungal culture reactor; K2, soil extracting reactor; M, flow-through cell; P, peristaltic pump; R, oxygen flow meter; S, stirring/heating plate; T, thermometer; W, moisturizing water. [Reprinted from May et al. (1997), copyright © with permission from the American Chemical Society.]

8.6.3   Compost Bioreactors

Composting is one of the most promising reactor systems for hazardous soil treatment. However, soil composing can be employed to treat soils contaminated by a variety of organic pollutants, such as PAHs, explosives, and pesticides. Composting can reduce the amount of extractable PAHs by stimulating biodegradation or binding of intermediate(s) to organic matter in soil. A compost bioreactor has been evaluated to enhance the removal of benzo[*a*]pyrene in contaminated soils by the white-rot fungus *Phanerochaete chrysosporium* (McFarland et al., 1992). The fungal system removed about 45% of the initial benzo[*a*]pyrene. The influence of compost bioaugmentation is also subject to removal of benzo[*a*]pyrene in contaminated soil using *P. chrysosporium* (McFarland and Qiu, 1995). The removal efficiencies were 62.8 ± 5.9 and $65.6\% \pm 1.2\%$ for fungal amended and unamended systems. In

these cases, bound residue formation is the only mechanism for the removal of benzo[*a*]pyrene in active compost systems.

8.6.4 Miscellaneous Bioreactors

Pilot-scale constructed wetlands are employed to treat PAH-contaminated water, and the role of fungi in these ecosystems has been examined (Giraud et al., 2001). A total of 40 fungal species belonging to 24 genera were identified from a contaminated wetland and a control wetland. Assay on liquid medium indicated efficient degradation of fluoranthene by 33 species and removal of 70% of anthracene by two species. New species included *Absidia cylindrospora*, *Cladosporium sphaerospermum*, and *Ulocladium chartarum*, which degrade high percentages of fluoranthene and anthracene. No correlation exists between the extracellular phenol oxidase activity and the degradative ability of fungi.

8.7 PAH DEGRADATION BY FUNGAL ENZYMES

Enzyme treatment provides a new strategy for the removal of PAHs in wastewaters and soils. The number of PAHs degraded by fungal enzymes continues to increase with ongoing research. White-rot Basidiomycetes are recognized to produce a complex inventory of enzymes, especially peroxidases. Certain white-rot fungi produce manganese–peroxidase (MnP) and laccase in the absence of lignin–peroxidase (LiP). White-rot fungi exhibiting this pattern include *Ceriporiopsis subvermispora* (Ruttimann et al., 1992; Ruttimann-Johnson et al., 1994), *Phlebia brevispora* (Ruttimann et al., 1992), *Panus tigrinus* (Maltseva et al., 1991), *Rigidoporus lignosus* (Galliano et al., 1991), *Stereum hirsutum*, and *Ganoderma valesiacum* (Nerud et al., 1991). Some fungi contain both types of enzymes, whereas others have one or the other. Most species express peroxidases and laccases as several isozymes (Lobos et al., 1994; Thurston, 1994; Johansson and Nyman, 1996; Munoz et al., 1997; Mester and Field, 1998). The glycosylated nature of both types of ligninolytic enzymes increases the stability of the enzymes. Nie et al. (1999) investigated the stability of recombinant and native peroxidases from *Phanerochaete chrysosporium*. One-electron oxidation by these enzymes produced cation radicals of the contaminants. Spontaneous chemical reactions such as C-C cleavage of hydroxylation of cation radicals resulted in the formation of more hydrophilic products (Hammel, 1995). Assimilation of these products and cometabolism in the presence of a suitable carbon source led to the formation of CO_2 by these fungi. The mechanism of action of these enzymes occurs due to a complex process of oxidation, reduction, methylation, and hydroxylation. Despite the ongoing research during the past decade, the oxidative mechanisms of these enzymes in PAH degradation are not clearly understood.

8.7.1 Peroxidase-Catalyzed Degradation

The extracellular peroxidases LiP and MnP can cleave several PAHs in vitro. LiP and MnP differ from each other in the range of reducing substrates. A compound as a substrate for LiP is determined by two factors: the size of the molecule and its redox potential. Little is known on the size of the compounds oxidized by LiP, for two reasons: nonconfirmation of the binding site(s) for reducing substrates and difficulty in the determination of kinetic parameters. Banci et al. (1999) tried to overcome these problems by employing a phenolic tetramer model compound.

LiP exhibits a higher redox potential than other peroxidases, suggesting a better oxidant of PAHs. However, all PAHs cannot be degraded by LiP. LiP is found to oxidize PAHs with an ionization potential (IP) of less than about 8 eV in the presence of H_2O_2 or H_2O_2-generating enzyme systems. In addition, compounds I and II differ in their ability to oxidize PAHs. Compound I can oxidize substrates of higher redox potential than compound II. The first, more recalcitrant substrate can be oxidized by the addition of a secondary substrate. The addition of veratryl alcohol (VA), a common secondary metabolite produced by white-rot fungi along with LiP (Jensen et al., 1994), enhances the rate and extent of PAH degradation (Vazquez-Duhalt et al., 1994; Goodwin et al., 1995). VA mediates with LiP to form cation radicals that react with the PAH. The oxidized VA cation may react with H_2O_2, producing a highly reactive dioxygen anion, which in turn reacts with the substrate. In other words, VA is an ideal reducing substrate for compounds I and II. Removal of PAHs becomes more efficient as the VA cation radical provides the oxidizing power of the active site to distant targets. Substrate-binding sites and catalysis and mediation by LiP and MnP have been discussed (Cameron et al., 2000; Mester and Tien, 2000). Table 8.3 lists the rates of degradation of PAHs and metabolic products by fungal peroxidases.

Limited knowledge is available on the successful oxidation of PAHs in vitro by MnP. MnP is unique, as it prefers its reducing substrate to be complexed Mn(II). It is established that MnP oxidizes Mn(II) to the powerful oxidizing agent Mn(III). A variety of PAHs are oxidized with IPs < 7.8 eV to acetoxy-PAH and PAH quinones by Mn(III) in acetate–water mixtures. A high yield of PAH quinones is recovered when incubated with extracellular fluids of white-rot fungi containing high levels of MnP (Field et al., 1992). One-electron oxidations of several PAHs by Mn(III) have been established, suggesting the role of MnP of *P. chrysosporium* in the degradation of PAHs in vivo (Field et al., 1993).

Small amounts of quinone products from anthracene and benzo[*a*]pyrene are produced during MnP/Mn(II) reactions by *Phanerochaete laevis* (Bogan and Lamar, 1996). MnP-based lipid peroxidation reactions revealed transformation of all four PAHs. Highly polar metabolites similar to diphenic acid (Moen and Hammel, 1994) are found during such reactions of phenanthrene,

TABLE 8.3 Peroxidase-Catalyzed Degradation of Polycyclic Aromatic Hydrocarbons and Metabolic Products

Fungus	Enzyme Conc.	PAH Conc.	Medium/Type of Bioreactor	Degradation/ Mineralization Rate (%)	Metabolic Products	Duration (minutes/ hours/days)	Reference
Phanerochaete laevis HHB-1625	MnP, 5 µl	Anthracene, 4µM Benzo[a]pyrene, 4µM	1.0-ml-vol. MnP/Mn(II) reactions	6 9	Quinone Quinone	30h	Bogan and Lamar, 1996
		Anthracene, 4µM Benzo[a]pyrene, 4µM Benz[a]anthracene, 4µM Phenanthrene, 4µM	1.0-ml-vol. MnP-based lipid oxidation system		Anthraquinone Ring-cleavage polar products Ring-cleavage polar products Ring-cleavage polar products	30 m	
Phanerochaete chrysosporium	MnP	Fluorene, 20µM $(1 \times 10^5$ dpm)	MnP-based lipid peroxidation system	>99	9-Fluorenone	7 d	Bogan et al., 1996a
Nematoloma frowardii	MnP, 1.96 U/ml	Anthracene, 10 mg/l Pyrene, 10 mg/l Fluoranthene, 10 mg/l Benzo[a]pyrene, 10 mg/l	1000-ml Erlenmeyer flasks	>99/3.7 CO_2 24/3.1 CO_2 13 54/0.4 CO_2		168h	Sack et al., 1997c
	MnP 1.96 U/ml +5 mM GSH	Anthracene, 10 mg/l Pyrene, 10 mg/l Fluoranthene, 10 mg/l Benzo[a]pyrene, 10 mg/l		>99/4.7 CO_2 >99 67 >99/4.0 CO_2	9,10-Anthraquinone		
Trametes versicolor	MnP	Phenanthrene, 10 mg/l Fluorene, 10 mg/l	High-N medium	75 100		11d 7d	Collins and Dobson, 1996
P. chrysosporium	LiP, 1 U	Benzo[a]pyrene, 50 nM			Benzo[a]pyrene 1,6-, 3,6-, and 6,12- quinones	120m	Haemmerli et al., 1986

benz[*a*]anthracene, and benzo[*a*]pyrene. Accumulation of anthraquinone occurs during oxidation of anthracene. MnP levels in *P. laevis*, like several other white-rot fungi, are strongly affected by the concentration of Mn(II) in the culture medium. This appears to be due to regulation at the level of mRNA transcription, a phenomenon revealed with the *mnp* genes of *P. chrysosporium* (Brown et al., 1990). Substantial losses of 12 three- to six-ring PAHs occurred during cultures and MnP-mediated lipid peroxidation reactions of *P. chrysosporium* (Bogan and Lamar, 1995). This suggests the participation of a one-electron oxidant stronger than LiP or Mn(III) in both systems. The oxidative cleavage of phenanthrene to diphenic acid by *P. chrysosporium* is a result of peroxidase-mediated lipid peroxidation (Hammel et al., 1992). A cascade of reactions producing numerous radical species during lipid peroxidation has been recognized (Buettner, 1993). Fluorene transforms to 9-fluorenone in vitro in a system containing Mn(II), unsaturated fatty acids, and using *P. chrysosporium* peroxidases or by purified recombinant MnP (Bogan et al., 1996a). The degradation of five individual PAHs and mineralization by MnP crude preparation of *Nematoloma frowardii* (1.96 U/ml) at 24°C have been demonstrated (Sack et al., 1997c). GSH is considered important for the degradation of certain PAHs with an IP > 7.55 eV. A possibility exists for the role of other "thio" compounds in the MnP-mediated oxidation of aromatic substrates. GSH-mediated reactions revealed enhanced mineralization of all PAHs. [^{14}C]Pyrene samples showed the highest amount of $^{14}CO_2$ release. This seems to be the first report on the enzymatic mineralization of PAHs. Paszczynski et al. (1985) reported the oxidation of GSH by the purified MnP of *P. chrysosporium*. This reaction produced H_2O_2, used by MnP to oxidize PAHs. The role of H_2O_2 to enhance anthracene degradation by *Bjerkandera* sp. strain BOS55 has been demonstrated (Kotterman et al., 1996).

One way to improve the dissolution rates of these compounds is by the use of surfactants. Combined treatment by Tween 80 and glucose oxidase of the extracellular fluid of 6-day-old *Bjerkandera* sp. strain BOS55 cultures revealed elevated daily rates of oxidation of anthracene and benzo[*a*]pyrene of 1450 and 450 mg/l, respectively (Kotterman et al., 1998a). PAHs were degraded in the presence of unsaturated fatty acids in the form of Tween 80, MnP, and Mn(II) (Moen and Hammel, 1994; Kapich et al., 1999). However, the role and mechanism of these additives in MnP catalysis are still unknown.

8.7.1.1   *Role of Miscible Solvents.* PAH solubility can be increased in water–solvent mixtures. The oxidation of anthracene can be enhanced in the presence of cosolvent using *Bjerkandera* sp. strain BOS55 (Field et al., 1995a). Solvent additions of 6 to 21% (v/v) acetone or ethanol increase anthracene oxidation (98 to 99.5%) to anthraquinone three times more than at 3% solvent. Anthraquinone formation is 4 to 12 times faster at 20% solvent than at 3% solvent.

Peroxidases are well known for their high tolerance to miscible solvents. This property can be exploited in the use of transformation of PAHs, as these compounds are solubilized in solvent–water mixtures. Ligninolytic peroxidases are known to tolerate 10 to 40% (v/v) miscible solvents. VA is oxidized by LiP isozyme H1 of *P. chrysosporium* in 30% and 40% (v/v) 2-methylethanol and 2-propanol at 30% and 20% of the control rate, respectively. MnP isozyme H4 from *P. chrysosporium* exhibited normal activity in 40% (v/v) 2-propanol (Hammel et al., 1993). PAH compounds were oxidized in vitro by LiP in the presence of miscible compounds such as 10 to 30% tetrahydrofurans, dioxane, dimethylformamide, acetonitrile, or 2-propanol (Hammel et al., 1986; Vazquez-Duhalt et al., 1994). LiP and MnP from extracellular fluid of 5-day *Bjerkandera* sp. strain BOS55 cultures and semipurified preparations from *P. chrysosporium* were used for their tolerance to miscible solvents and in vitro conversion of anthracene in solvent–water mixtures (Field et al., 1996b). MnP assayed with 2,6-dimethoxyphenol showed 20% retention of normal activity in the presence of 45 to 58% (v/v) acetone, 31 to 34% (v/v) ethanol, and 29% (v/v) 2-propanol. LiP assayed with VA were two to three times less tolerant than MnP to solvents. This was the first report of H_2O_2-enhanced degradation of anthracene by MnP in the presence of 40% (v/v) acetone. MnP also oxidized anthracene in the absence of exogenous H_2O_2 at a lesser rate at acetone concentrations from 5 to 60%. The optimum acetone concentration is 10% (v/v) for LiP-mediated oxidation of anthracene. The toxicity of solvents to LiP increases in the preferential order 2-methoxyethanol < methanol < ethanol < acetone < 2-propanol. Biotransformation by MnP is several times slower than the dissolution kinetics in 40% (v/v) acetone. The toxicity of solvents to MnP increases in the preferential order 2-propanol < ethanol < acetone.

8.7.1.2 *Influence of Cyclodextrins.* PAHs can be solubilized in organic solvents, but these are generally not compatible with enzyme activity. Cyclodextrins can be employed for PAH solubilization instead of solvents. The most common forms are α-, β-, and γ-cyclodextrins containing six, seven, and eight glucose units, respectively. These can result in the formation of inclusion complexes with water-insoluble compounds by attaching these molecules to their hydrophobic activity. High concentrations of 2-hydroxypropyl-β-cyclodextrin (hpβCD) were incorporated in water-insoluble 1,1′-dimethylferrocene and tetrathiafulvalene. The solubility of naphthalene and benzo[*a*]pyrene was enhanced by hpβCD about 224 and 7500 times, respectively (Male et al., 1995). A distinct correlation exists between PAH solubility and hpβCD in terms of aromatic rings in the PAH. LiP oxidation of cyclodextrin-included substrates is similar to that reported by Hammel et al. (1986) and Haemmerli et al. (1986) for mixed solvent systems. Of several PAHs, LiP oxidizes only anthracene, pyrene, and benzo[*a*]pyrene complexed with hpβCD. The hpβCD solubilizes PAHs better than solvent systems using LiP for the oxidation of PAHs.

8.7.2 Laccase-Catalyzed Degradation

Laccases are copper-containing proteins that catalyze the oxidation of a large variety of aromatic and nonaromatic compounds. Laccases catalyze one-electron oxidations with the formation of radicals that undergo subsequent nonenzymatic reactions. Laccase is considered to oxidize only compounds that have a low redox potential. Oxidation of numerous PAHs without a reactive functional group by laccase is rather astounding. Extracellular laccases from submerged cultures of strains of *Coriolus versicolor*, *Panus tigrinus*, *Phlebia radiata*, and *P. tremellosa* and cultures of strains of *P. tigrinus*, *P. radiata*, and *Agaricus bisporus* on wheat straw during solid-state fermentation have been purified (Leontievsky et al., 1997). Laccases from submerged cultures are blue in color and exhibit characteristic absorption and electron paramagnetic resonance (EPR) spectra, and those of solid-state fermentation are yellow-brown and exhibit atypical EPR spectra and no blue oxidase spectra. Blue and yellow-brown forms of laccases show high homology based on N-terminal amino acid sequences.

In recent years, fungal laccases have been reviewed a number of times. The enzymology and electron transfer mechanism of laccases have been described (Messerschmidt, 1993; Solomon et al., 1996). Messerschmidt (1997) edited a book on laccases that contained a series of articles on laccase kinetics, mechanisms of action, and the role of these enzymes. Insights into the molecular mechanisms of copper proteins can be gained by spectroscopic techniques (Solomon et al., 1998; Randall et al., 2001). Genes for numerous laccases have been cloned and the sequences deposited in the appropriate gene register. The occurrence, catalysis, and applications of laccases in bioremediation have recently been discussed (Mayer and Staples, 2002). Despite good research during the last decade, little is known as to the kinetic properties of fungal laccases. The precise mechanism of laccases in the degradation of PAHs is still not understood. Laccase-mediated degradation of PAHs and metabolic products is noted in Table 8.4.

Laccase of *Trametes versicolor* oxidized most of 14 PAHs in vitro (Majcherczyk et al., 1998). 1,2-Acenaphthenedione and 1,8-naphthalic acid anhydride were detected during oxidation of acenaphthylene with laccase of *T. versicolor* (Johannes et al., 1998). Only 35% of anthracene was transformed stoichiometrically to 9,10-anthraquinone after 72 hours of incubation in vitro by laccase of *T. versicolor* (Johannes et al., 1996). Crude preparation of laccase of *T. versicolor* oxidized nearly 24% anthracene after 24 hours (Collins et al., 1996). Purified laccase isozymes laccase I and II from *T. versicolor* also displayed a limited amount of oxidation. The major product of anthracene oxidation in these reactions was anthraquinone. Nearly 0.35 and 0.44 per mole of anthracene was removed by LiP and MnP of *P. chrysosporium* with molar yields of formation of anthraquinone (Field et al., 1996b).

TABLE 8.4 Laccase-Catalyzed Degradation of Polycyclic Aromatic Hydrocarbons and Metabolic Products

Fungus	Laccase Conc./ Mediator	PAH Conc.	Medium/ Type of Bioreactor	Degradation/ Mineralization Rate (%)	Metabolic Products	Duration (minutes/ hours)	Reference
Trametes versicolor	Laccase, 4 U/ml	Acenaphthylene, 25 μM	10-ml reaction tubes	37		72h	Majcherczyk et al., 1998
		Anthracene, 25 μM		18			
		Benzo[*a*]pyrene, 25 μM		19			
		Eight other PAHs, 25 μM		10			
	Laccase, 4 U/ml + HBT, 1 mM	Acenaphthene, 25 μM		99	1,2-Acenaphthenedione, 1,8-naphthalic acid anhydride		
		Acenaphthylene, 25 μM		100	1,2-Acenaphthenedione, 1,8-naphthalic acid anhydride		
		Anthracene, 25 μM		99	9,10-Anthraquinone		
		Benzo[*a*]pyrene, 25 μM		91	1,6-, 3,6-, 6,12-benzo[*a*] pyrene-diones, quinone		
		Fluorene, 25 μM		92	9-Fluorenone		
		Perylene, 25 μM		96	Polymeric products, quinone		
T. versicolor	Laccase, 4 U/ml	Acenaphthene	10-ml reaction tubes	3		70h	Johannes et al., 1998
		Acenaphthylene		35	1,2-Acenaphthenedione, 1,8-naphthalic acid anhydride		
	Laccase, 4 U/ml + HBT, 1 mM	Acenaphthene		100	1-Hydroxyacenaphthene, 1-acenaphthenone, 1,8-naphthalide		
		Acenaphthylene, 25–100 μM		100	*cis*- and *trans*-1, 2-Dihydroxyacenaphthene, 1-acenaphthenone, 1,8-naphthalic acid anhydride, 1,2-acenaphthenedione		

TABLE 8.4 *Continued*

Fungus	Laccase Conc./Mediator	PAH Conc.	Medium/Type of Bioreactor	Degradation/Mineralization Rate (%)	Metabolic Products	Duration (minutes/hours)	Reference
T. versicolor	Laccase, 4 U/ml	Anthracene, 15 mg/l	10-ml reaction tubes	35	9,10-Anthraquinone	72h	Johannes et al., 1996
	Laccase, 4 U/ml + ABTS, 1mM	Anthracene, 15 mg/l		64	9,10-Anthraquinone		
	Laccase, 4 U/ml + HBT, 1mM	Anthracene 15 mg/l		97	9,10-Anthraquinone, two coupling products		
T. versicolor	Laccase, 4 U/ml	Anthracene, 20 mg/l	30-ml serum bottles	24	9,10-Anthraquinone	24h	Collins et al., 1996
	Laccase I + ABTS	Anthracene, 20 mg/l		84	9,10-Anthraquinone		
	Laccase II + ABTS	Anthracene, 20 mg/l		70	9,10-Anthraquinone		
	Laccase + ABTS	Benzo[*a*]pyrene, 20 mg/l		84			
	Laccase I + ABTS,	Benzo[*a*]pyrene, 20 mg/l		100			
	Laccase II + ABTS, 4 U/ml + 1mM	Benzo[*a*]pyrene, 20 mg/l		90			
Pycnosporus cinnabarinus	Laccase, 8000 U + 2mM ABTS	Benzo[*a*]pyrene, 0.1mM	Bench-scale reactor	95	Benzo[*a*]pyrene 1,6-, 3,6- and 6,12-quinones	24h	Rama et al., 1998
Pleurotus eryngii	300mU/ml + 1mM HBT	Benzo[*a*]pyrene, 50μM	Sodium–acetate buffer	70		24h	Rodriguez et al., 2004
Coriolopsis gallica	Laccase	Anthracene, 20 μM	10-ml reaction tubes				Pickard et al., 1999
	5 U + 1mM ABTS			100		9h	
	5 U + 1mM HBT			80		9h	
	5 U + 1mM ABTS + 1mM HBT			100		10m	

8.7.2.1 Laccase Bioreactors. Laccase treatment of benzo[*a*]pyrene was conducted in a bench-scale reactor (Rama et al., 1998) containing a 2-L buffer supplemented with Tween 20 and 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS). Acetone was used as a solvent for benzo[*a*]pyrene. Laccase was added and incubated at 30ºC for 24 hours with light stirring by a continuous bubble of air. All experiments were performed in 10-ml reaction tubes with a buffer (Johannes et al., 1996, 1998; Majcherczyk et al., 1998). Samples of laccase and PAH dissolved in acetone were added to each tube. Mediators dissolved in acetone were then added. Reaction tubes were tightly closed and incubated on a horizontal shaker at room temperature for a specified period of time. All experiments were performed in 2-ml reaction volumes in 30-ml serum bottles containing laccases in different forms (Collins et al., 1996). Laccase titer is adjusted by dilution with buffer. PAHs are dissolved in acetone, and Tween 60 is added to increase PAH bioavailability. Mediators are also added and bottles are flushed with 100% oxygen for 5 minutes and incubated for 24 hours at 27ºC.

8.7.2.2 Influence of Mediators. Application of laccases in the presence of mediator compounds has been established to enhance the degradation of PAHs. These are low-molecular-weight compounds that result in high and novel oxidation capacities. Laccase–mediator systems were originally developed to overcome problems of biobleaching of wood pulps. ABTS acts as a cooxidant that can interact with laccase to accomplish electron transfer. ABTS is chemically oxidized in two steps via $ABTS^+$ and $ABTS^{2+}$ with respective redox potentials of 0.68 and 1.09 V (Scott et al., 1993). VA, anisyl alcohol, and benzyl alcohol can be better oxidized by $ABTS^{2+}$ than by $ABTS^+$ (Bourbonnais et al., 1998; Majcherczyk et al., 1999). Moreover, the catalytic formation of $ABTS^{2+}$ by laccase is unknown at present. ABTS acts as a mediator in a thermodynamically unfavorable reaction such as $ABTS^+$ cation radical + VA ↔ ABTS + VA cation radical (Majcherczyk et al., 1999).

Three metabolites, benzo[*a*]pyrene 1,6-, 3,6-, and 6,12-quinones in a 2:1:1 ratio, are produced during transformation of benzo[*a*]pyrene by purified laccase of *Pycnosporus cinnabarinus* in the presence of ABTS (Rama et al., 1998). Addition of ABTS stimulates the oxidation of anthracene highly by two purified laccase isozymes of *T. versicolor*, and ABTS is essential for the oxidation of benzo[*a*]pyrene (Collins et al., 1996). Anthraquinone is identified as a major metabolite of isozyme oxidation of anthracene. This type of one-electron mechanism appears to be similar to being catalyzed by LiP from *P. chrysosporium* in the oxidation of anthracene (Hammel et al., 1991). *Pleurotus eryngii* degrades benzo[*a*]pyrene only in the presence of mediators (Rodriguez et al., 2004). Nearly 70% degradation of benzo[*a*]pyrene occurs in the presence of HBT; however, ABTS provides 60% degradation. Purified laccase from a strain of *Coriolopsis gallica* oxidized fluorene in the presence of HBT and ABTS without the formation of oxidation products (Bressler et al., 2000). Laccase of *T. versicolor* removed acenaphthene and

acenaphthylene completely in the presence of HBT after 70 hours (Johannes et al., 1998). Several metabolic products have been identified by oxidation of a laccase–HBT system. Radical cations were produced during the primary step in the oxidation of acenaphthene and acenaphthylene. 1,2-Acenaphthenedione (XII) and 1,8-naphthalic acid anhydride (XIII) were major metabolites and constituted 50% of the metabolites obtained after 70 hours. The pathway for the oxidation of acenaphthene and acenaphthylene by a laccase–HBT system is shown in Figure 8.9.

Addition of HBT to the reaction mixture causes almost complete elimination of acenaphthene, acenaphthylene, fluorene, anthracene, benzo[*a*]pyrene, and perylene by laccase of *T. versicolor* (Majcherczyk et al., 1998). Stoichiometric transformation of fluorene to 9-fluorenone was obtained similar to oxidation of anthracene to 9,10-anthraquinone. Laccase–HBT oxidation of [^{14}C]benzo[*a*]pyrene results into three main products of quinones: 1,6-, 3,6-, and 6,12-benzo[*a*]pyrene-diones. Electrochemical oxidation of benzo[*a*]pyrene also produced the formation of three similar quinones (Jeftic and Adams, 1970). *T. versicolor* produced a heat-stable natural factor of ≤10kDa that plays a role in the laccase-mediated oxidation of anthracene (Collins et al., 1996). At present, the role of this natural mediator compound in the laccase-mediated degradation of PAHs is not clear. Majcherczyk and Johannes (2000) studied coupling of pyrene–polyethylene glycol (5000) in a laccase-mediator system. Substrate and laccase were separated by a membrane to prevent direct contact. Laccase oxidized the low-molecular-weight mediators HBT and ABTS to radicals that permeated the membrane and reacted with the pyrene–polyethylene glycol model compound. This resulted in two main oxidation products, and the same products are produced in a reaction without a membrane.

8.7.3 Miscellaneous Enzymes

Chloroperoxidase (CPO) from *Caldariomyces fumago* is the most versatile halogenating enzyme in the hemoprotein family (Yi et al., 1999). CPO is able to perform a broad range of chemical reactions. It is a H_2O_2-dependent chlorinating enzyme and catalyzes peroxidase-, catalase-, cytochrome P450-type reactions of dehydrogenation, H_2O_2 decomposition, and oxygen insertion, respectively. CPO from *C. fumago* utilized 17 of 20 aromatic hydrocarbons as substrates in the presence of H_2O_2 and chloride ions (Vazquez-Duhalt et al., 2001). The reaction rates were from 0.6 per minute for naphthalene to 758 per minute for 9-methylanthracene. The CPO-mediated reactions of aromatic hydrocarbons resulted in the formation of mono-, di-, and trichlorinated compounds. The metabolites of acenaphthene, anthracene, phenanthrene, fluorene, and pyrene were dichloro- and trichloroacenaphthene, 9,10-dichloroanthracene, chlorophenanthrene, dichlorofluorene, and chloro- and dichloropyrene, respectively. Interestingly, the recalcitrant and carcinogenic five- and six-ring PAHs are also substrates for CPO-mediated reactions.
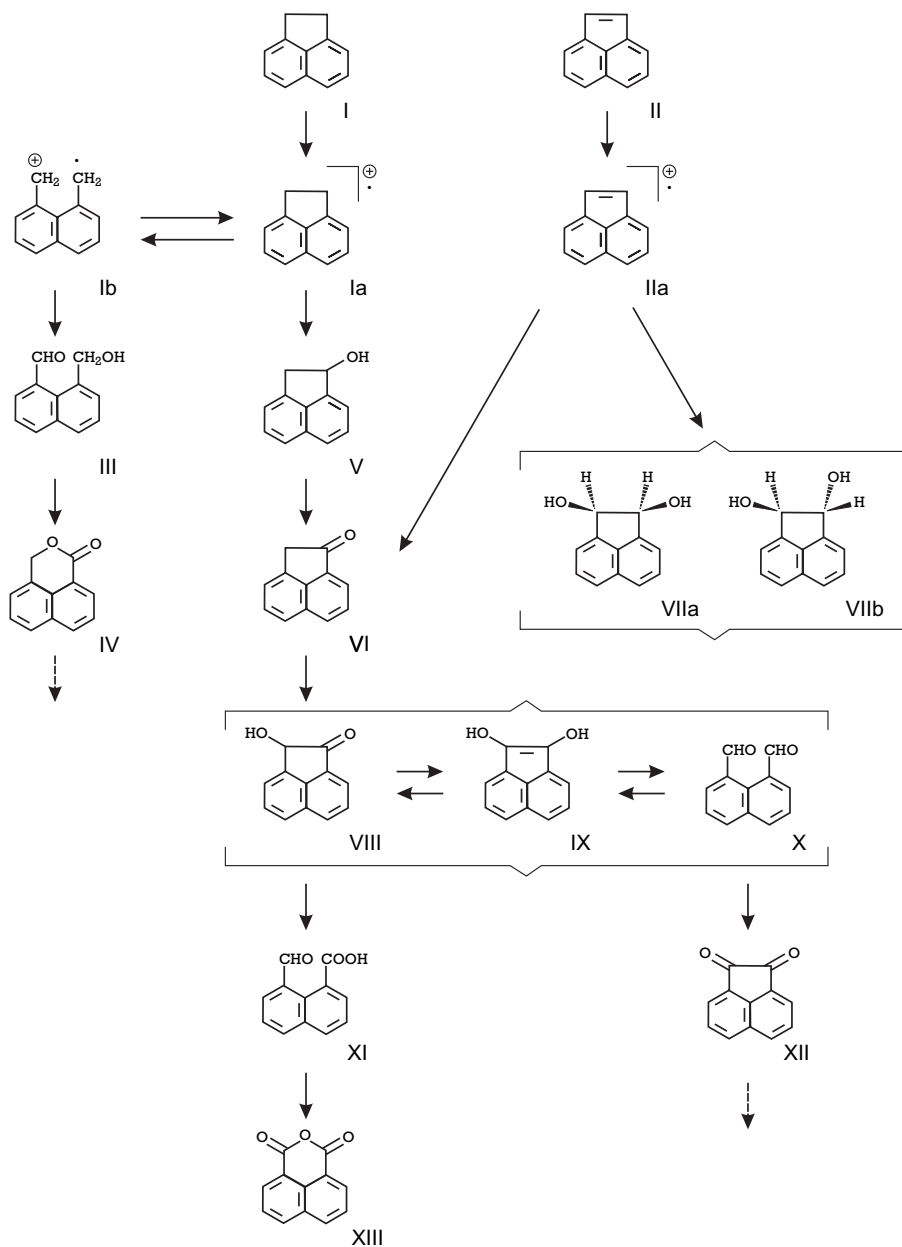
Figure 8.9 Tentative pathway for oxidation of acenaphthene and acenaphthylene by *Trametes versicolor* laccase/HBT system; the compounds expected but not detected by GC-MS analysis are indicated within braces. I, acenaphthene; Ia, radical cation; Ib, benzyl radical cation; II, acenaphthylene; III, hydroxyaldehyde; IV, 1,8-naphthalide; V, 1-hydroxyacenaphthene; VI, ketone 1-acenaphthenone; VIIa and VIIb, *cis*- and *trans*-1,2-dihydroxyacenaphthene; VIII, 1-hydroxy-2-acenaphthenone; IX, 1,2-hydroxyacenaphthylene; X, 1,8-naphthalic aldehyde; XI, 1,8-naphthalaldehydic acid; XII, 1,2-acenaphthenedione; XIII, 1,8-naphthalic acid anhydride. [Reprinted from Johannes et al. (1998), copyright © with permission from Elsevier.]

In addition to LiP and MnP, a third ligninolytic peroxidase, called the versatile peroxidase (VP), is recognized in *Pleurotus* and *Bjerkandera* (Ruiz-Duenas et al., 2001). Two of these VPs have been cloned, sequenced, and characterized. VPs show the existence of several substrate oxidation sites. VP from *Pleurotus eryngii* degraded 40% of benzo[*a*]pyrene after 5 hours (Rodriguez et al., 2004). Hydrogen peroxide appeared to be a limiting factor, as the percentage of benzo[*a*]pyrene did not increase after 5 hours.

8.8   CYTOCHROME P450 IN DEGRADATION OF PAHs

Cytochrome P450 is a superfamily of enzymes that plays an important role in the oxidation of xenobiotic compounds. Limited studies are known on fungal P450, due to difficulty in isolating active enzyme and to a low content in some species. In filamentous fungi, *Aspergillus ochraceus* (Ghosh et al., 1983), and *Neurospora crassa* (Kapoor and Lin, 1984), benzo[*a*]pyrene hydroxylase activity is induced by benzo[*a*]pyrene. The use of cytochrome P450 inhibitors, the requirement for isolated enzyme on NADPH or cytochrome P450 reductase (CPR), and the incorporation of ^{18}O in the substrate are indirect evidence for the involvement of P450. Kellner et al. (1997) discussed the natural P450s, catalytic sites and crystal structures, electron transfer, and engineering P450 by random mutagenesis and for bioremediation. Four decades of knowledge of cytochrome P450 has been summarized by Omura (1999).

Cytochrome P450 plays an important role in PAH degradation by fungi. Initial oxidation of PAHs by cytochrome P450 monooxygenase resulted into an arene oxide that is further catalyzed by epoxide hydrolase to form a *trans*-dihydrodiol (Cerniglia, 1997). The CPR gene is isolated from two widely studied species, *Cunninghamella elegans* and *C. echinulata* (Yadav and Loper, 2000). The *C. elegans* CPR gene is obtained by screening a genomic library using PCR and primers. The 2420-bp coding region contains two apparent introns, of 149 bp and 138 bp. Northern blot analysis indicated that the CPR gene is expressed transcriptionally in *C. elegans* and appears to be inducible by an alkane substrate, *n*-tetradecane. Phylogenetic comparison of the *C. elegans* CPR deduced (710aa) indicates that it is more closely related to animal CPR than to yeast and plants. A 2074-bp sequence containing most of the CPR gene homolog from *C. echinulata* was also isolated. Cytochrome P450 systems in fungi related to PAH degradation have been described by van den Brink et al. (1998). *P. chrysosporium* metabolized phenanthrene to phenols and *trans*-dihydrodiols via a cytochrome-mediated reactions (Sutherland et al., 1991). Screening of a number of fungi from petroleum-contaminated soils identified *Penicillium janthinellum* as a highly potent species that exhibits P450-mediated biotransformation of pyrene (Launen et al., 1995). Moreover, the formation of 9-hydroxybenzo[*a*]pyrene from oxidation of benzo[*a*]pyrene by 50% of the fungal isolates yields a different pattern

of quinones, shown by ligninase of *P. chrysosporium* (Haemmerli et al., 1986). Screening of several saprophytic and plant pathogenic Basidiomycetes found *Crinipellis stipitaria* to be one of the most potent pyrene-degrading strains (Lambert et al., 1994; Lange et al., 1994; Wunder et al., 1994).

Pleurotus ostreatus metabolizes several PAHs, similar to nonligninolytic fungi (Bezalel et al., 1996b, c). *P. ostreatus* oxidizes phenanthrene to *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene. One atom of $^{18}O_2$ is incorporated in this product, and the inhibitor of P450 monooxygenase reduces the formation of this product. These results show that cytochrome P450 monooxygenase is involved in an initial oxidation of phenanthrene. Laccase may be involved during the pathway of PAH products at a later stage. A microsomal and cytosolic cytochrome P450 system has been identified, catalyzing hydroxylation of benzo[*a*]pyrene in *Pleurotus pulmonarius* (Masaphy et al., 1995). The presence of cytochrome P450 in the mycelium of *P. chrysosporium* and P450-mediated hydroxylation of benzo[*a*]pyrene in both microsomal and soluble fractions have been demonstrated in vitro (Masaphy et al., 1996). A type I binding spectrum is produced by both P450 fractions on addition of benzo[*a*]pyrene. Hydroxylation was NADPH-dependent and inhibited by carbon dioxide. The K_m value was 89 µm for microsomal P450 and 400 µm for cytosolic P450. The presence of PAH-degrading cytochrome P450 and exocellular ligninolytic enzymes in one fungus is something unique. A cytochrome P450 gene is identified and cloned in *P. chrysosporium* by reverse transcription (RT)-PCR using a set of degenerate primers (Kullman and Matsumura, 1997). The amino acid sequence deduced for the partial protein was compared to other known cytochrome P450 sequences and indicated that this is a first member of a new family of cytochrome P450, designated as CYP63-1A. Northern blot analysis suggested the expression of CYP63-1A under both nitrogen-deficient and nitrogen-rich culture conditions.

8.9 FUNGAL DEGRADATION OF PAHs IN SOILS

During the past decade, remediation has focused on biodegradation in soils of high-molecular-weight PAHs, which have recalcitrant properties. A critical factor in soil remediation is the temporal bioavailability of PAHs. The refractory fraction of benzo[*a*]pyrene increased twice within 3 months after initial contamination (Field et al., 1995b). More rapid degradation of contaminants was found in artificially or newly polluted soils than in aged contaminated soils. The aging of contamination reduces the bioavailability fraction that can be attributed to adsorption in micropores and/or changes in binding forms (e.g., oxidative coupling reactions).

Most earlier bioremediation research employed indigenous bacteria for PAH degradation. Four pathways have been recognized for the bioremediation of PAH-contaminated soils, depending on the soil structure, organic matter, and type of contamination or prevailing microflora (Mahro et al.,

1994). On-site treatability and case studies on PAH degradation have been discussed by Wilson and Jones (1993). However, bacterial methods exhibit several drawbacks, due to problems of bioavailability in soils of heavier PAHs. One way to overcome this problem is to use white-rot fungi. These organisms produce extracellular enzymes, which can be effective with contaminants of low bioavailability and thus are beneficial for the process of bioremediation.

The ability of several white-rot fungi to degrade a wide variety of xenobiotics has been established. The unique properties of these fungi has been revealed. The degradation of PAHs by white-rot fungi has been investigated extensively under a variety of conditions in liquid cultures, but the applicability of this system in contaminated soils has also been demonstrated by several researchers. The degradation of PAHs in liquid cultures, soils, and reactors by different white-rot fungi under various experimental conditions during the 1990s has been reviewed by Canet et al. (1999). Notably, there were significant differences between the degradation capabilities in liquid cultures, solid-state fermentations, and soil bioremediation. Dense mycelial growth and efficient enzyme production were necessary to eliminate PAHs from contaminated soils.

Several advantages of white-rot fungal technology are known over those of other biological and conventional treatments. One problem when inoculating contaminated soil with white-rot fungi can be competition from other strains of fungi and indigenous bacteria. Individual degradation activity between indigenous flora and selected fungi in soils is difficult to estimate. White-rot fungi can be pregrown on a suitable solid substrate that is later used for inoculation of the contaminated soil. Synergistic degradation by white-rot fungi and bacteria can also occur in the bioremediation of PAH-contaminated soil. Protocols need to be developed to enhance the final removal of contaminants in soil. Monitoring of $^{14}CO_2$ production is important for mineralization, and toxicity assays must be incorporated into the procedures used to monitor the effectiveness of PAH mycoremediation. The kinetics of *Cunninghamella echinulata* var. *elegans* is credited with determining the degradation rates of PAH-contaminated soils related to three supplemental nutrient solutions (Cutright, 1995). Table 8.5 shows the transformation of PAHs in soils by fungi.

8.9.1   Influence of Cosubstrates and Surfactants

To survive in soil, white-rot fungi need a substrate. However, successful degradation of high-molecular-weight PAHs by white-rot fungi depends on an ample supply of a suitable carbon cosubstrate, such as wood chips and peat (Lamar and Glaser, 1994), wheat straw (Andersson and Henrysson, 1996; in der Wiesche et al., 1996; Wolter et al., 1997; Martens et al., 1999; Canet et al., 2001; Rodriguez et al., 2004; Mollea et al., 2005), rice, annual plant stems and wood (Field et al., 1995b), and bark (Eggen and Sveum, 1999). The extent of benzo[*a*]pyrene degradation was improved by the use of hemp stem

TABLE 8.5 Fungal Transformation of Polycyclic Aromatic Hydrocarbons in Soils

Fungus	PAH Conc.	Cosubstrate/ Pretreatment	Degradation/ Mineralization Rate (%)	Metabolic Products/ Enzyme Activity	Duration (months/ weeks/days)	Reference
Pleurotus ostreatus	Creosote-contaminated soil (four-ring PAHs)	Reference soil Bark pretreatment Bark pretreatment and temperature	30 40 59		2 m	Eggen and Sveum, 1999
P. ostreatus	Aged creosote-contaminated soil (benzo[a]pyrene) 12.40 ppm	Potato pulp, 15–20% and sawdust from *Picea* sp.	32		3 m	Eggen and Majcherczyk, 1998
	Artificially added [14C]benzo[a]pyrene, 100 mg/1082 g soil		49 1 14CO₂		2 m	
Phanerochaete chrysosporium	Aged creosote-contaminated soil Total PAHs, 1175 mg/kg Benzo[a]pyrene, 28.6 mg/kg		96 94		4 m	Holroyd and Caunt, 1994
Bjerkandera sp. strain BOS55	Benzo[a]pyrene, 100 mg/kg	Rice	80	1500 nM/ml·per minute MnP peak titer, lower MIP titer	22 d	Field et al., 1995b
Chrysosporium lignorum	Benzo[a]pyrene	Several substrates	2 CO₂		115 d	Morgan et al., 1993

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TABLE 8.5 Continued

Fungus	PAH Conc.	Cosubstrate/ Pretreatment	Degradation/ Mineralization Rate (%)	Metabolic Products/ Enzyme Activity	Duration (months/ weeks/days)	Reference
P. ostreatus	Aged creosote-contaminated soil (three-ring PAHs)	Substrate, soil–substrate, fish oil	>73	Variable low laccase activity	7 w	Eggen, 1999
	Total PAHs, 1909 mg/kg Three-ring PAHs, 1113 mg/kg	Fish oil and spent mushroom compost	86 89		7 w	
	Four-ring PAHs, 696 mg/kg		87			
	Five-ring PAHs, 59 mg/kg		48			
Absidia cylindrospora	Fluorene, 100 μg/g	Soil–slurry	98		12 d	Garon et al., 2004
Pleurotus sp. Florida	8 PAHs, 50 μg [14C]Pyrene, 14 kBq [14C]Benz[a]anthracene, 33 kBq	Wheat straw	75 53 $^{14}CO_2$ 25 $^{14}CO_2$		15 w	Wolter et al., 1997
	[14C]Benzo[a]pyrene, 50 kBq in the presence of eight unlabeled PAHs, 50 μg		39 $^{14}CO_2$			
Pleurotus sp. Florida	[14C]Pyrene, 300 μg and 15 kBq	5% Milled wheat straw	25–40 $^{14}CO_2$		25 w	Martens et al., 1999
	[14C]Benz[a]anthracene, 300 μg and 33 kBq	5% Milled wheat straw	15–24 $^{14}CO_2$			
	[14C]Benzo[a]pyrene, 300 μg and 56 kBq	5% Milled wheat straw	13–34 $^{14}CO_2$			

Organism	Compound	Substrate	% / $^{14}CO_2$	Metabolite	Time	Reference
Dichomitus squalens *Pleurotus* sp.	[^{14}C]Pyrene, 50µg and 17 kBq	Milled wheat straw	13–39 $^{14}CO_2$		15 w	in der Weische et al., 1996
P. ostreatus and *P. sajor-caju*	Anthracene, 100 mg/kg Benz[*a*]anthracene, 20 mg/kg Dibenz[*a,h*]anthracene, 20 mg/kg	Wheat straw	40–50 $^{14}CO_2$ 99 92 31	9,10-Anthracene-dione Benz[*a*]anthracene-7, 12-dione	65 d	Andersson and Henrysson, 1996
P. chrysosporium *Kuehneromyces mutabilis* *Agrocybe aegerita*	Anthracene, 100 mg/kg [^{14}C]Pyrene, 1 µ labeled and unlabeled 5 mg/68.5 g	Wheat straw Sterilized soil Soil inoculation Sterilized soil Soil inoculation	98 5.1 $^{14}CO_2$ 47.7 $^{14}CO_2$ 1.5 $^{14}CO_2$ 38.5 $^{14}CO_2$	9,10-Anthracene-dione	63 d	Sack and Fritsche, 1997
P. chrysosporium	Anthracene, 400 ppm	Marshan sandy loam	65	9,10-Anthraquinone	27 d	Bogan et al., 1996c
P. chrysosporium	[^{14}C]Phenanthrene, 50–54 kBq	Nonsterile inoculated soil	38 $^{14}CO_2$	Soluble and bound compounds	21 d	Brodkorb and Legge, 1992
Irpex lacteus	Anthracene, 50 ppm Phenanthrene, 50 ppm Fluoranthene, 50 ppm Pyrene, 50 ppm	Milled wheat straw	49 37 25 52	Low LiP, laccase	3 m	Novotny et al., 2000
P. ostreatus strains	Anthracene, 50 ppm Pyrene, 50 ppm Phenanthrene, 50 ppm	Milled wheat straw	81–87 84–93 41–64	Anthraquinone, low MnP, laccase	2 m	Novotny et al., 1999
P. ostreatus f6 strain	Pyrene, 10 µg/g Benzo[*a*]pyrene, 10 µg/g Benz[*a*]anthracene, 10 µg/g Benzo[*b*]fluoranthene, 10 µg/g	Milled wheat straw	85 79 64 49		15 w	Eichlerova et al., 2000

TABLE 8.5 Continued

Fungus	PAH Conc.	Cosubstrate/Pretreatment	Degradation/Mineralization Rate (%)	Metabolic Products/Enzyme Activity	Duration (months/weeks/days)	Reference
Isolate B13	Pyrene, 10µg/g		86			
	Benzo[a]pyrene, 10µg/g		75			
	Benz[a]anthracene, 10µg/g		52			
	Benzo[b]fluoranthene, 10µg/g		35			
Isolate B26	Pyrene, 10µg/g		90			
	Benzo[a]pyrene, 10µg/g		78			
	Benz[a]anthracene, 10µg/g		62			
	Benzo[b]fluoranthene, 10µg/g		42			
Phanerochaete sordida	Creosote-contaminated soil (3-, and 4-ring PAHs), 4017 µg/g	Soil inoculation	75		8 w	Lamar and Glaser, 1994
P. sordida	Creosote-contaminated soil, Three-ring PAHs Four-ring PAHs	Wood chips	85–95 24–72		60 d	Davis et al., 1993
Coniothyrium (Co)	Aged contaminated soil, 0.8g PAH/kg	Air-dried soil Soil inoculation	26.5		1 m	Potin et al., 2004a
Fusarium F5			27.5			
Cladosporium sphaerospermum	Aged contaminated soil, total PAHs	Nonsterile soil	23		4 w	Potin et al., 2004b
P. chrysosporium	Naphthalene, 600 mg/kg	Sterilized soil Nitrogen supply	75		80 d	Mollea et al., 2005

wood as a cosubstrate instead of rice (Field et al., 1995b). Glucose (Ravelet et al., 2001) and glucose and several plant-derived carbon sources (Morgan et al., 1993) were also tested. Straw was found to be the best substrate to support the colonization of soil and mineralization of benzo[*a*]pyrene in soil by different white-rot fungi. *Irpex lacteus* efficiently colonized sterile and nonsterile soil by exploratory mycelium from a wheat straw inoculum (Novotny et al., 2000). *Trametes versicolor*, *Kuehneromyces mutabilis*, *Laetiporus sulphureus*, and *Agrocybe aegerita* mineralized 15.5%, 5%, 10.7%, and 3.7% of phenanthrene, respectively, within the same period during cultivation on wheat straw (Sack et al., 1997b). Cultivation in straw cultures revealed the mineralization of [^{14}C]pyrene by *K. mutabilis* (6.7%), *L. sulphureus* (4.3%), and *A. aegerita* (3.3%); *T. versicolor* exhibited the highest mineralization (34.1%). Thirteen (13) basidiospore-derived isolates of *P. ostreatus* f6 strain were cultivated, differing in the level of production of MnP and laccase and morphological features on natural substrates (Eichlerova et al., 2000). The ligninolytic enzymes showed substantially higher activities in straw than in liquid culture.

Fish oil contains a mixture of triglycerides and other constituents and is a versatile and innocuous product for many applications. In certain applications, biodegradable fish oil can be replaced by synthetic surfactants. Fish oil is added to spent mushroom compost and then mixed with creosote-contaminated soil (Eggen, 1999). During bioaugmentation of artificially contaminated soil in soil-slurry applications, *Absidia cylindrospora* degraded 98% of fluorene within 288 hours (Garon et al., 2004). The native microflora degraded only 54% of fluorene. Addition of maltosyl cyclodextrin (MCL) improved the enhancement of fluorene biodegradation during the first 144 hours. MCL also enhanced fluorene degradation up to 80% by native microflora after 288 hours.

Some studies have also focused on artificially contaminated soils (Morgan et al., 1993; McFarland and Qiu, 1995; Andersson and Henrysson, 1996; Eggen and Majcherczyk, 1998). In *P. chrysosporium*, the degradation of xenobiotics is inhibited by nitrogen concentration above a certain level (Bumpus et al., 1985). Certain fungal species [e.g., *P. ostreatus* (Majcherczyk and Huttermann, 1993; Morgan et al., 1993) and *B. adusta* (Kaal et al., 1993; Kotterman et al., 1994)] also degraded xenobiotics in the presence of nitrogen. So these species can be used to remediate contaminated soil rich in nitrogen, or it is necessary to add nitrogen during the mycoremediation process. During the use of white-rot fungi for bioremediation, the availability of fungal inoculum is important so that it can withstand competition from native microflora and subsequently, penetrate the surrounding soil. Spent mushroom culture can be advantageous for the fungal inoculum (Buswell, 1994). Another point concerns how the inoculum is applied. A comparison have been made of two different commercial sources of inoculum and the influence of organizing soil and substrate in layers or in a homogenized mix (Eggen, 1999). The fluorescein diacetate (FDA) hydrolyzing and laccase activities were measured in

soil to correlate enzyme activity in the substrate with the degradation rate of PAHs.

In certain cases it is important to eliminate the most easily degradable PAHs by stimulating bacteria before inoculation with white-rot fungi. This pretreatment may enhance subsequent degradation of the recalcitrant PAHs by white-rot fungi. In this way, extracellular enzymes can be utilized properly. Microbial activity is stimulated at low temperatures by pretreatment with fertilizer and PAH degradation is enhanced without the addition of fungi (Eggen and Sveum, 1999).

Surfactants are known to be effective in enhancing bioavailability in soils by increasing the dissolution rate and solubility of PAHs. Solubilization occurs above a specific threshold, the critical micelle concentration (CMC), when surfactant molecules aggregate to form micelles. Surfactants can lower the interfacial tension between the solid and aqueous phases, thereby enhancing the mobilization of soil-bound PAHs. Solubilization depends on the type and dose of surfactant, surfactant–soil interactions, the hydrophobicity of PAHs, and the duration of contact between PAHs and soil. Nonionic polyoxyethylene (POE) surfactants are found beneficial for the bioremediation of PAHs. Factors such as surfactant toxicity and solubilizing efficiency are considered in the selection of surfactants for soil bioremediation. In one study, nonionic POE surfactants influenced the elimination of nine PAHs in a contaminated soil slurry by *P. chrysosporium* (Zheng and Obbard, 2001). Nonionic surfactants, Tween 80 and Tween 40, and the anionic surfactant sulfonol have been found to enhance the activities of ligninolytic enzymes during solid-phase fermentation of *Pleurotus floridae* and *Phellinus igniarius* (Dombrovskaya and Kostyshin, 1996). Surfactants have also been used to determine the nature of transformation products in *P. ostreatus*–inoculated soil microcosms (Bogan et al., 1999).

8.9.2   Fate of PAH Disappearance

In fungi, biodegradation of PAHs is known to be the major mechanism of removal from soils. Formation of nonextractable polymerized bound residues of PAHs by fungi has also been demonstrated.

8.9.2.1   PAH Degradation and Mineralization.   White-rot fungi have the ability to facilitate the removal of PAHs from creosote. All PAHs except heterocyclic compounds in aged creosote-contaminated soil are degraded significantly by inoculation of *Pleurotus ostreatus* (Eggen and Sveum, 1999). The pretreatments include a bark temperature fertilizer (BTF) treatment soil and a bark temperature (BT) treatment soil. Adding bark and incubating at 22°C before inoculation causes better four-ring PAH degradation (59%) than does no pretreatment or pretreatment with fertilizer. Pretreatment without fertilizer addition (BT) offers almost twice the PAH degradation rates as those of pretreatment with fertilizer (BTF). In aged creosote-contaminated

soil, *P. ostreatus* removed benzo[*a*]pyrene extensively (28%) during the first month, and further incubation produced additional degradation of only 4% (Eggen and Majcherczyk, 1998). Elimination of artificially added [^{14}C]benzo[*a*]pyrene was higher than the aged benzo[*a*]pyrene originally present. Benzo[*a*]pyrene was eliminated in creosote-contaminated soil up to 30%, 47%, and 94% after 1, 2, and 4 months, respectively, by *P. chrysosporium* (Holroyd and Caunt, 1994). However, the total PAHs were lowered to 96% after 4 months. Medium-sized polyethylene columns were employed for the degradation of PAHs in aged creosote-contaminated soil by *P. ostreatus* (Eggen, 1999). Each column was a unique pile design related to substrate and soil. The experiments show variable laccase activity in soil and do not correlate positively with the rate of degradation. Eight highly condensed unlabeled and three ^{14}C-labeled PAHs were degraded during solid-state fermentation by *Pleurotus* sp. *florida* (Wolter et al., 1997). High rates of degradation of benzo[*a*]pyrene, benz[*a*]anthracene, and benzo[*g,h,i*]perylene have been achieved. The highest degradation occurred in pyrene within the first 5 weeks at all concentrations. In a 15-week period, *Pleurotus* sp. *florida* mineralized 53% [^{14}C]pyrene, 25% [^{14}C]benz[*a*]anthracene, and 39% [^{14}C]benzo[*a*]pyrene to $^{14}CO_2$ in the presence of eight unlabeled PAHs. Mineralization was independent of the initial concentration of benzo[*a*]pyrene. More than 40% wheat straw substrate was degraded by the fungus. *Pleurotus* sp. *florida* has been shown to have the ability to grow from straw substrate into soil (Martens and Zadrazil, 1998). *Pleurotus* sp. *florida* mineralized [^{14}C]heavier PAHs in soils (Martens et al., 1999). This reveals a great potential to degrade PAHs in sand in the presence of straw amendment without reduction in bioavailability. All four species of *Pleurotus* showed a low degree of mineralization in solid-state cultures after 4 weeks using wheat straw (Rodriguez et al., 2004).

Soil microorganisms enhance the mineralization of [^{14}C]pyrene with a *Pleurotus* sp. irrespective of the time of application (in der Wiesche et al., 1996). The *Pleurotus* sp. showed high [^{14}C]pyrene mineralization by soil application. *Phanerochaete chrysosporium* showed synergistic behavior with indigenous soil microorganisms in the oxidation of low-molecular-weight PAHs in soil slurry (Zheng and Obbard, 2002a). However, the fungus enhanced the oxidation of PAHs up to 43%. Limited oxidation of high-molecular-weight PAHs such as chrysene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene, and benzo[*g,h,i*]perylene occurs due to low solubility and/or slow diffusion through contaminated soil. When PAHs are dissolved in acetone and added to cultures, the extent of PAH removal is a direct function of PAH solubility in the preferential order phenanthrene > pyrene > benzo[*a*]pyrene. On the contrary, efficient oxidation of pyrene and benzo[*a*]pyrene takes place in the presence of surfactant, Tween 80, with recoveries of less than 16.3% and 0.35%, respectively. In general, five white-rot fungi show a significant reduction in anthracene and benz[*a*]anthracene, and a small decrease in dibenz[*a,h*]anthracene in aerated soils (Andersson and Henrysson, 1996). Significantly greater proportions of

9,10-anthracene-dione and benz[*a*]anthracene-7,12-dione have been identified in soil during treatment with *Pleurotus* and *Phanerochaete* than with *Trametes*. Breakdown of 90% and 80% of three- and four-ring PAHs, respectively, occurred during treatment of PAH-contaminated soil for 2 months by *P. chrysosporium* (Baud-Grasset et al., 1993).

During soil inoculation, *Kuehneromyces mutabilis* and *Agrocybe aegerita* exhibited 47.7% and 38.5% rates of pyrene mineralization (Sack and Fritsche, 1997). Based on the total mineralization and bound residue formation, *K. mutabilis* removed about 50% and 75% of pyrene in sterilized and nonsterilized soils, respectively. *K. mutabilis* enhanced up to 42% of pyrene removal compared to native soil microflora. In organic-rich sterile soils, 12.6%, 37.9%, and 9.4% of five PAHs were removed by groups of wood- and straw-degrading, terricolous, and ectomycorrhizal fungi, respectively (Gramss et al., 1999). Degradation of three- and four-ring PAHs was not enhanced in organic-rich nonsterile soils inoculated with wood- and straw-degrading fungi. Degradation of five- and seven-ring PAHs was 29 to 42% based on the optimum combinations of fungal species and soil type. Degradation was delayed by the same fungi in organic-poor nonsterile soil. Indigenous bacteria were killed by mycelia of *Pleurotus*, whereas *Hypholoma* and *Stropharia* supported the development of bacteria in soil, not necessarily for PAH degradation. PAHs were degraded irrespective of their molecular size in organic-rich and wood-chip-amended soils. *Phialophora alba* increased pyrene degradation by 9% compared to indigenous microorganisms, but pyrene metabolism was inhibited by the addition of glucose (Ravelet et al., 2001). *Mucor racemosus* var. *sphaerosporus* was not efficient in freshwater sediment bioremediation with or without the addition of glucose, as it reduced the rate of pyrene degradation by native microflora. The ergosterol content was not correlated with pyrene degradation. Four white-rot fungi (i.e., *P. chrysosporium*, *P. ostreatus*, *C. versicolor*, and Wye isolate no. 7) were shown not to be able to thrive and remove PAHs in highly contaminated soil, as they remained in a metabolically inactive form (Canet et al., 2001). The autochthonous microflora revealed the greatest potential for PAH degradation. Mineralization of [^{14}C]phenanthrene was 20% in nonsterile soil with native microflora, and mineralization increased to 38% by the addition of *P. chrysosporium* at 38ºC after 21 days (Brodkorb and Legge, 1992). In the presence of *P. chrysosporium*, the proportion of soluble and bound polar compounds increased. These polar products are supposed to be more readily metabolized by native soil microflora. In sterile soil, degradation activity was decreased several times by the addition of *P. chrysosporium*. Pyrene degradation was suppressed by *P. chrysosporium*, native microflora, and other abiotic characteristics of soil (Tucker et al., 1995).

Irpex lacteus removed three- and four-ring PAHs efficiently in an artificially spiked soil after 3 months (Novotny et al., 2000). The fungus produced LiP and laccase but no MnP in sterile soil. The extracellular enzyme production and PAH in soil have been eliminated by mycelia of white-rot fungi

(Novotny et al., 1999). Tube reactors are used for the cultivation of white-rot fungi in soil. The activities of fungal MnP and laccase are 5 to 20 times higher in coarse-milled wheat straw than in soil. The enzyme activities in *P. ostreatus* and *T. versicolor* are similar in soil compared to low levels in *P. chrysosporium*. Ligninolytic enzymes showed higher activities in milled wheat straw than in liquid culture by isolates of *P. ostreatus* (Eichlerova et al., 2000). The ability to decompose the straw was very good after 50 days and a high loss of organic mass was attained. All seven PAHs were degraded successfully by the original *P. ostreatus* strain f6 and isolates B13 and B26 in soil samples after 15 weeks. No correlation existed between higher or lower rates of production of ligninolytic enzymes (laccase and MnP) and the extent of degradation. This suggests the involvement of some other factors in the biodegradation process. Growth of six basidiospore-derived isolates in nonsterile soil indicates their suitability for the exploitation in biotechnological research.

Isolates of the genera *Mucor*, *Cladosporium*, *Coniothyrium*, *Fusarium*, *Sphaeropsis*, and *Trichoderma* revealed degradation rates of aged PAH-contaminated soil above 20% (Potin et al., 2004a). *Coniothyrium* (Co) and *Fusarium* (F5) exhibited 20%, 22%, and 30% of three-, four-, and six-ring PAH, respectively. Except for benzo[*b+k*]fluoranthene and dibenz[*a,h*]anthracene, high-molecular-weight PAHs were degraded at an average rate of 40 to 50%.

8.9.2.2 Bound Residue Formation.

*8.9.2.2 **Bound Residue Formation.*** PAHs are also incorporated into soil organic matter, resulting in the formation of bound residue formation. Some of this knowledge was discussed earlier in the section on bioreactors. Burgos et al. (1996) reported on free radical–mediated copolymerization of pollutant and/or degradation products and the organic precursors of humic and fulvic acids by extracellular fungal oxidoreductases due to covalent incorporation of pollutant into soil organic matter. This has been demonstrated in vitro for PCP (Ruttimann-Johnson and Lamar, 1996), for other chlorophenols (Sarkar et al., 1988; Lassen et al., 1994), for trinitrotoluene (Dawel et al., 1997), and for certain herbicides (Kim et al., 1997). The formation of nonextractable polymerized residues of PAH has been demonstrated by white-rot fungi in mixtures of sand and fungal-colonized straw (Wolter et al., 1997). A binding-desorption model for the humic substances for remediation has also been discussed (Rebhun et al., 1996).

Some humification of anthracene, fluoranthene, and benzo[*a*]pyrene in two PAH-contaminated soils was shown to occur during growth by *P. ostreatus* (Bogan et al., 1999). Significant increases in the amount of radioactivity associated with the humic acid and humic fractions were found in benzo[*a*]pyrene-contaminated soils by *P. ostreatus*. The removal of PAHs was enhanced significantly in both soils by *P. ostreatus* in the presence of surfactants and related amendments, but humification was not always enhanced.

8.9.3   Factors Affecting Biodegradation of PAHs

A number of factors affect successful mycoremediation in soils. Mycodegradation depends on the inherent properties of PAHs and soils. Adsorption or covalent binding of PAHs to organic carbon, such as clay minerals and their particle size, affect the bioavailability, transport, fungal activity, and mycodegradation in the environment. A number of mechanisms, such as chemical binding, hydrogen bonding, cation bridging, ion exchange, covalent bonding, ligand exchange, and van der Waals forces, may be involved in the process of sorption, although these mechanisms are not completely understood. Environmental parameters (i.e., soil moisture, pH, redox potential, oxygen, nutrient content, and temperature) also influence the rate of degradation. The extent of contaminant retention is related directly to the octanol–water (K_{ow}) partitioning coefficient and the amount of organic matter in the soil. These parameters are usually interrelated and can be optimized to improve fungal degradation.

Different factors are known to affect the degradation of PAHs in soils by white-rot fungi (Boyle et al., 1998). *T. versicolor* produces a range of unidentified polar metabolites from [^{14}C]benzo[*a*]pyrene along with low mineralization in liquid culture. Addition of soil, sawdust, or ground alfafa to a liquid culture inhibits the formation of polar metabolites with little effect on mineralization. Nitrogen sources have little effect on the formation of polar metabolites, but inhibit mineralization. Radiolabeled polar metabolites in soil are more rapidly mineralized by soil microorganisms than by benzo[*a*]pyrene. Degradation in soil is increased by some surfactants, thus increasing benzo[*a*]pyrene concentration in the soil moisture phase. Bioremediation of PAH-contaminated soils with *P. chrysosporium* can be improved coupled with the regulation of carbon, nitrogen, pH, and so on (Brodkorb and Legge, 1992).

In selected fungal experiments, Canet et al. (1999) described a temperature range 20 to 39°C, with or without O_2 content, a 4-day to 15-week period of incubation, nutrient contents, cosubstrates, and surfactants in a liquid medium, artificially contaminated soil, and bioreactors, respectively. *P. chrysosporium* grows very fast at an optimum temperature of 30°C and sporulates easily in culture. About a 75% increase in the rate of degradation occurs during incubating the soil at 30°C rather than at 20°C (Field et al., 1995b). Increasing the benzo[*a*]pyrene in soil from 100 mg/kg to 1000 mg/kg doubles the initial daily rate of degradation to 21 mg/kg. Degradation of all PAH groups increases nearly 1.4- to 3.6-fold when the temperature increases from 8°C to 22°C (Eggen and Sveum, 1999). In general, degradation is 12 to 20% at 8°C except for heterocyclic compounds (40%). At both temperatures, four-ring PAHs exhibit more degradation than three-ring PAHs. In general, higher degradation occurs with pretreated soil inoculated at 8°C. Unexpectedly high rates of degradation are noted for heterocyclic compounds at 8°C and in soil pretreated without fertilizer. A three-factor model depicted the same trend

in the effect of pretreatment at 8ºC and for the interaction between pretreatment and temperature. Porous pumice stones added to the soil also provide adequate aeration and high rates of degradation. Benzo[*a*]pyrene degraded at a daily rate of 0.8 mg/kg in the presence of a 100% O_2 atmosphere when the soil was inoculated with *P. chrysosporium* (Qui and McFarland, 1991). An air atmosphere was shown to be adequate for PAH degradation during the creation of rapid O_2 mass transfer from a high liquid/gas surface area ratio (Kotterman et al., 1994). Slightly open lids of jars were shown to ensure aeration of microcosm experiments (Canet et al., 2001). In each microcosm, the moisture content was maintained at 50% of the water-holding capacity, and each was incubated at 22ºC. Continuous aeration of 3 to 6 ml/min with sterile CO_2-free moistened air has been used with flasks incubated at 25ºC (in der Wiesche et al., 1996; Wolter et al., 1997).

8.10   FUNGAL METABOLISM OF COMPLEX PAH MIXTURES

PAH mixtures degraded rapidly, within 4 to 8 hours, and no compound remained after 24 hours using *C. elegans* (Pothuluri et al., 1995). About 54% of benzo[*a*]pyrene was degraded in 4 hours, 66% by 8 hours, and complete degradation by 24 hours. More rapid degradation of 84% and 85% has been found in phenanthrene and fluoranthene, respectively, in 4 hours, 95% for each by 8 hours, with complete degradation by 24 hours. About 88% pyrene was degraded by 4 hours, 97% by 8 hours, with complete degradation by 24 hours. Hydroxylated metabolites formed within 24 hours, exhibiting spectra similar to those of 3-(8-dihydroxyfluoranthene) β-glucopyranoside, phenanthrene 3,4-dihydrodiol, 2-hydroxy-1-phenanthryl β-D-glucopyranoside, 3-fluoranthene β-glucopyranoside, and 3-hydroxybenzo[*a*]pyrene. These metabolites are similar to those formed during studies of individual PAH compounds. A Coelomycete, *Cyclothyrium* sp., degrades 59%, 70%, 74%, and 38% of anthracene, phenanthrene, pyrene, and benzo[*a*]pyrene, respectively, in a mixture after 192 hours of incubation (da Silva et al., 2003).

White-rot fungi are also suggested for the biodegradation of complex mixtures containing various PAHs in crude oil–, coal tar–, and creosote-polluted sites. *P. chrysosporium* has been proved to degrade all compounds in a mixture in creosote (Kennes and Lema, 1994). Phenol and cresol are first eliminated, followed by PCP and the PAHs. Metabolites such as naphthoquinone and anthraquinone accumulated. Individual PAH is degraded slightly faster at similar initial concentrations than the same compounds in the mixture. The PAH components of anthracene oil disappeared after incubating with *P. chrysosporium* under nitrogen-limiting culture conditions (Bumpus, 1989).

Fungal enzymes are also used for the degradation of complex mixtures of PAHs. A PAH mixture was biodegraded by 1.96 U/ml MnP crude preparation of *Nematoloma frowardii* in the presence of GSH within 168 hours (Sack

et al., 1997c). No anthracene or phenanthrene was detected after 96 hours. More than 75% each of pyrene and fluoranthene and more than 30% each of benz[*a*]anthracene, benzo[*a*]pyrene, and benzo[*b*]fluoranthene were degraded. Phenanthrene disappeared due to a combination of volatilization and degradation. Chrysene was not degraded. All anthracene, 65% of pyrene, 40% of fluoranthene, and 20% of benzo[*a*]pyrene was degraded in the absence of GSH. Chrysene, benz[*a*]anthracene, and benzo[*b*]fluoranthene were not degraded without GSH. Selectivity of LiP to oxidize either anthracene or benzo[*a*]pyrene has been demonstrated when mixed with pyrene (Male et al., 1995). In a PAH mixture of benzo[*a*]pyene and pyrene, pyrene is oxidized only after complete assimilation of benzo[*a*]pyrene. Similarly, in a mixture of anthracene and pyrene, pyrene was oxidized only after complete assimilation of anthracene. A lag period was noted before the formation of pyrene oxidation products, which suggests a faster rate of oxidation of pyrene than for either anthracene or benzo[*a*]pyrene alone.

Substrate interactions have been evaluated by biodegradation kinetics of naphthalene, phenanthrene, and pyrene in sole-substrate systems and in binary and ternary mixtures (Guha et al., 1999). Aerobic batch aqueous systems inoculated with mixed culture were employed. In sole-substrate experiments, Monod kinetic parameters and yield coefficients were calculated from substrate depletion and release of CO_2. The ternary mixture revealed the inhibition of biodegradation of naphthalene and enhancement of biodegradation of phenanthrene and pyrene. A multisubstrate Monod kinetic model predicted substrate interactions in binary and ternary mixtures based on the parameters of sole-substrate experiments. Simulations of biomass growth kinetics throw sufficient light on various range behaviors in PAH mixtures. Substrate interactions may be large due to the presence of a large number of compounds in PAH-contaminated environments. It has been shown that single, binary, and ternary PAH mixtures can be solubilized by three different anionic surfactants (Chun et al., 2002).

8.11   PAH DEGRADATION BY FUNGAL–BACTERIAL CO-CULTURES

Fungi and bacteria exhibit different pathways for the catabolism of PAHs. Both present certain disadvantages with regard to degradation of PAHs at the sites. Fungi and bacteria can be employed together or in different phases to maximize the extent of the biodegradation. Bacteria are generally unable to hydroxylate PAH compounds. Fungal transformation of PAHs, followed by bacterial degradation of polar metabolites, can be an effective strategy for PAH metabolism. Some fungal–bacterial co-cultures studies have proven its potential for the detoxification and mineralization of PAHs. In one study, significant degradation and microbial growth occurred on chrysene, pyrene, benz[*a*]anthracene, benzo[*a*]pyrene, and dibenz[*a,h*]anthracene in basal

salts medium (BSM) in the presence of a combination of fungal–bacterial co-cultures of *Stenotrophomonas maltophilia* VUN 10010 and a bacterial consortium of VUN 10009 and *Penicillium janthinellum* VUO 10201 (Boonchan et al., 2000). These co-cultures mineralized 25% of benzo[*a*]pyrene to CO_2 after 49 days, along with temporary accumulation and disappearance of metabolites. Inoculation of fungal–bacterial co-cultures resulted in 53% mineralization of [^{14}C]benzo[*a*]pyrene to $^{14}CO_2$ in 100 days. Biodegradation of benzo[*a*]pyrene by the same fungal–bacterial co-cultures has also been reported by Stanley et al. (1999). *P. janthinellum* was not able to utilize benzo[*a*]pyrene on the BSM. However, 16 to 64% of the ^{14}C label was identified as polar metabolites in the presence of cosubstrate (i.e., pyrene, glucose or malt extract, yeast extract, peptone, dextrose medium). Benzo[*a*]pyrene was mineralized by *S. maltophilia* and bacterial consortia. Radioactive recovery of 13 to 32% pyrene (250 mg/l) was achieved as $^{14}CO_2$. *P. janthinellum* and *S. maltophilia* resulted in 58% of benzo[*a*]pyrene mineralization with 61% water-soluble products after 56 days on media containing pyrene (250 mg/l) and benzo[*a*]pyrene (50 mg/l).

Nearly 8.5% $^{14}CO_2$ in fungal cultures and 73% water-soluble metabolites was recovered from [^{14}C]benzo[*a*]pyrene after 15 days using *Bjerkandera* sp. strain BOS55 (Kotterman et al., 1998b). Many unidentified polar metabolites accumulated. Further incubation of up to 215 days accounts for a small increase in mineralization (13.5%). Inocula of indigenous microflora prepared from activated sludge, acid forest soil, and a culture grown on 2,2′-diphenic acid, when added to fungal cultures, enhanced benzo[*a*]pyrene mineralization by 20 to 27% by day 56. Also, the amount of polar metabolites decreased from 69% to 37–48%. Further incubation for 215 days resulted in the recovery of 34% $^{14}CO_2$ and 16% polar metabolites. Studies by Kotterman et al. (1998b), Stanley et al. (1999), and Boonchan et al. (2000) resulted in significant reduction in the mutagenic potential of cultures or soil extracts as observed in a *Salmonella typhimurium* revertant assay. These studies also reveal that fungal oxidation can enhance benzo[*a*]pyrene mineralization by bacteria. A similar phenomenon was also noted for pyrene (in der Wiesche et al., 1996).

Phenanthrene degradation correlates to microbial density and biomass increase, the highest biomass reaching 238 and 50 mg/l for *Rhodotorula glutinis* and *Pseudomonas aeruginosa*, respectively (Romero et al., 1998). *R. glutinus* is as active as *P. aeruginosa* during growth on phenanthrene in liquid mineral basal medium.

8.12   BIOTECHNOLOGY AND BIOENGINEERING

Genetically engineered microorganisms have shown potential in applications for bioremediation in soil, groundwater, and activated sludge environments, due to the enhanced degradative capabilities of a wide range of contaminants.

Applications of genetically engineered microorganisms have been discussed in bioremediation, including risk assessment for the release of recombinant microorganisms, survival in a harsh world, and bioprocess monitoring and control (Sayler and Ripp, 2000). Several hundred genetic systems can be exploited for developments useful in bioremediation (Sayler et al., 1998; Menn et al., 2000). Strains and protein engineering can be designed for improved bioremediation. Genetic alterations can be designed and controlled via metabolic engineering. The present limited knowledge of biotechnology related to PAHs is focused on white-rot fungi, especially *P. chrysosporium*, and is quite complex. Multiple isozymes are produced in submerged cultures, and at least 10 structural genes for LiP isozymes have been designated, from *lipA* through *lipJ* in *P. chrysosporium* (Gaskell et al., 1994). LiP isozymes show some kinetic and substrate range differences, but the reason for the multiplicity of *lip* genes is unknown at present. LiP isozymes exhibit variations based on kinetics (Glumoff et al., 1990). In certain isozymes, access to the heme group is less restricted, due to the flexibility of the surrounding protein. Some of these variations may be due to differences in the oxidation–reduction potential of the various isoforms (Millis et al., 1989) or variations in active-site structures (Sinclair et al., 1995). LiP isozyme profiles have been prepared from liquid culture at the level of protein (Dosoretz and Grethlein, 1991; Boominathan and Reddy, 1992) and mRNA transcripts (Stewart et al., 1992; Moukha et al., 1993).

Lamar et al. (1995) purified mRNA from fungus-colonized soil with competitive reverse transcription-PCR (RT-PCR) to understand the physiological state of *P. chrysosporium* in soil. Transcripts of two LiP genes (*lipA* and *lipC*), two cellobiohydrolases (*cbh1-1* and *cbh1-4*), and mRNA encoding the mitotic spindle protein β-tubulin in PCP-contaminated soil by *P. chrysosporium* have been detected. Three MnP isozyme genes in *P. chrysosporium* were expressed during a bench-scale PAH soil bioremediation experiment using competitive RT-PCR (Bogan et al., 1996b). The expression of three MnP genes is coordinately regulated under growth conditions. The degradation of fluorene and disappearance of chrysene in soil during an early phase of fungal colonization correlated with the degree of expression of MnP genes in *P. chrysosporium*.

The constant oxidation of anthracene that occurred throughout a 25-day experiment suggested the involvement of several LiP isozymes of *P. chrysosporium* (Bogan et al., 1996c). The mRNA from soil was extracted for the expression of 10 *lip* genes in anthracene-transforming soil cultures and quantification of *lip* mRNA by competitive RT-PCR. The levels of extractable *lipA* transcript and protein LiP H8 were well correlated. An unusual pattern of gene expression was observed. The early phase of fungal growth was dominated by *lipA* and *lipD*. Transcripts of *lipJ* reached the highest levels with any gene during later stages of growth. The high levels of *lipA* mRNAs were maintained for 3 weeks and outnumbered transcripts of several other *lip* genes (i.e., *lipB*, *lipG*, *lipH*, and *lipJ*) throughout the study. The gene expres-

sion of *lip* under different liquid conditions suggests an early phase of carbon limitation, followed by a transition to nitrogen starvation. Thus, anthracene transformation occurs by mechanisms different from those involved in the oxidation of non-LiP substrate PAHs.

8.13   CONCLUSIONS AND FUTURE PERSPECTIVES

Over the past two decades, extensive knowledge has been developed on the biodegradation of PAHs by fungi, especially by white-rot fungi. In soil systems, fungi have demonstrated their ability to degrade two- or three-ring PAHs faster, but their activity to degrade PAHs with four or more fused rings is limited. In general, two- or three-ring PAH compounds have half-lives on the order of days rather than months or years for five- or higher fused ring PAHs. Thus, it is important to screen, isolate, and apply fungi that can degrade high-molecular-weight PAHs faster in field soils.

Fungal degradation of PAHs has been achieved successfully in bioreactors despite limited work. However, methods to remove toxic materials and improve PAH bioavailability and biodegradation need to be developed to make mycoremediation more effective. Good controls are required to minimize abiotic losses. Factors controlling fungal acclimatization to maintain prolonged activity in bioreactors are yet to be determined. The limited biotreatment of exogenous enzymes in mycodegradation of PAHs has been recognized with good success. High titers of LiP and MnP activities in white-rot fungi are necessary to carry out the mineralization of PAHs. The ability to produce highly active radicals and H_2O_2 in the presence of mediators by *Nematoloma frowardii* MnP indicates the possibility of using this method in the bioremediation of soils, sediments, or wastewaters contaminated with high-molecular-weight PAHs (Sack et al., 1997c). Natural mediators can be discovered and used to amplify the reactions of MnP.

The use of white-rot fungi in soil bioremediation of PAHs is an exciting and promising technology. However, it is still difficult to formulate a protocol for the design of the mycoremediation system. *Pleurotus* sp. Florida is proposed as a promising candidate for soil bioremediation because of its ability to degrade highly condensed PAHs, its high tolerance to substrates, and a large capacity for competing with indigenous soil microflora (Wolter et al., 1997; Martens and Zadrazil, 1998; Martens et al., 1999). *Irpex lacteus* is also considered as a suitable fungus for soil remediation, due to the production of different ligninolytic enzymes, robust growth, its ability to colonize soil, and its resistance to the inhibitory action of soil microflora (Novotny et al., 2000). A better understanding of the microbial ecology of white-rot fungi in highly contaminated field soils is important before the development of mycoremediation strategies. The Heterobasidiomycetes and Hyphomycetes are involved in a natural selection process in PAH-contaminated habitats (Kurtzmann and Fell, 1998). PAH oxidation can be optimized by indigenous fungi in designing

improved techniques for the bioremediation of contaminated soils. Factors controlling PAH bioavailability and mycodegradation and their optimization are also necessary to enhance mycoremediation in field soils. A possibility exists for the use of *P. chrysosporium* to treat highly naphthalene-contaminated soil in a landfarming process (Mollea et al., 2005).

At present, targets for cleanup of PAH contamination by fungi are not clear. Complete removal by fungi of PAHs in contaminated soils is not known. The formation, mechanism, and removal of nonextractable residues from PAH during fungal biodegradation and humification in soils are important issues for the future. The fate and toxicity of PAH carbon skeletons integrated in humic substances are yet to be determined.

In recent years, the use of fungal–bacterial co-cultures has demonstrated the detoxification of PAHs. Such studies have opened new horizons on the processes of fungal biodegradation. Bioaugmentation is one approach to soil bioremediation and has been used on a contaminated site when not enough of the appropriate microorganisms are present. To date, studies on the role of fungal bioaugmentation at PAH-contaminated sites are unknown. No doubt, fungal–bacterial co-cultures and bioaugmentation are employed to overcome limited metabolic capabilities, but these also suffer from certain limitations. Little is known on the biodegradation of complex mixtures of PAHs by fungi. The preferential oxidation of one PAH over the other in a mixture will be a topic of great interest in the future.

Despite extensive work on fungal detoxification of the PAHs, the metabolic pathways have not been fully elucidated. The principal types of metabolites are identified and the search continues. Mycodegradation and toxicity of the oxidation products of PAHs and their interactions with the soil matrix open new vistas of future research. An understanding of metabolic pathways and metabolites is crucial to the development of successful strategies for mycoremediation in soils. Extensive research on the enzymes involved in metabolic pathways is necessary to optimize the process of biodegradation by fungi. Genes encoding enzymes involving degradation of high-molecular-weight PAHs must be cloned, sequenced, and characterized. The development of selective bioengineered fungi can open a new era in PAH metabolism.

REFERENCES

Alfani, A., G. Maisto, M.V. Prati, and D. Baldantoni (2001) Leaves of *Quercus ilex* L. as biomonitors of PAHs in the air of Naples (Italy). *Atmos. Environ.* **35**: 3553–3559.

Andersson, B.E., and T. Henrysson (1996) Accumulation and degradation of dead-end metabolites during treatment of soil contaminated with polycyclic aromatic hydrocarbons with five strains of white-rot fungi. *Appl. Microbiol. Biotechnol.* **46**: 647–652.

Arbuckle, J.G., M.E. Bosco, D.R. Case, E.P. Laws, J.C. Martin, M.L. Miller, et al. (1991) *Environmental Law Handbook*, 11th ed. Government Institutes, Inc., Rockville, MD.

Banci, L., S. Ciofi-Baffoni, and M. Tien (1999) Lignin and Mn peroxidase-catalyzed oxidation of phenolic lignin oligomers. *Biochemistry* **38**: 3205–3210.

Banerjee, D.K., P.M. Fedorak, A. Hashimoto, J.H. Masliyah, M.A. Pickard, and M.R. Gray (1995) Monitoring the biological treatment of anthracene-contaminated soil in a rotating-drum bioreactor. *Appl. Microbiol. Biotechnol.* **43**: 521–528.

Barclay, C.D., G.F. Farquhar, and R.L. Legge (1995) Biodegradation and sorption of polyaromatic hydrocarbons by *Phanerochaete chrysosporium. Appl. Microbiol. Biotechnol.* **42**: 958–963.

Baud-Grasset, F., S. Baud-Grasset, and S.I. Safferman (1993) Evaluation of the bioremediation of a contaminated soil with phytotoxicity tests. *Chemosphere* **26**: 1365–1374.

Bezalel, L., Y. Hadar, and C.E. Cerniglia (1996a) Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus. Appl. Environ. Microbiol.* **62**: 292–295.

Bezalel, L., Y. Hadar, P.P. Fu, J.P. Freeman, and C.E. Cerniglia (1996b) Metabolism of phenanthrene by the white rot fungus *Pleurotus ostreatus. Appl. Environ. Microbiol.* **62**: 2547–2553.

Bezalel, L., Y. Hadar, P.P. Fu, J.P. Freeman, and C.E. Cerniglia (1996c) Initial oxidative products in the metabolism of pyrene, anthracene, fluorene, dibenzothiophene by the white rot fungus *Pleurotus ostreatus. Appl. Environ. Microbiol.* **62**: 2554–2559.

Bogan, B.W., and R.T. Lamar (1995) One-electron oxidation in the degradation of creosote polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **61**: 2631–2635.

Bogan, B.W., and R.T. Lamar (1996) Polycyclic aromatic hydrocarbon-degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. *Appl. Environ. Microbiol.* **62**: 1597–1603.

Bogan, B.W., R.T. Lamar, and K.E. Hammel (1996a) Fluorene oxidation in vivo by *Phanerochaete chrysosporium* and in vitro during manganese peroxidase-dependent lipid peroxidation. *Appl. Environ. Microbiol.* **62**: 1788–1792.

Bogan, B.W., B. Schoenike, R.T. Lamar, and D. Cullen (1996b) Manganese peroxidase mRNA and enzyme activity levels during bioremediation of polycyclic aromatic hydrocarbon-contaminated soil with *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **62**: 2381–2386.

Bogan, B.W., B. Schoenike, R.T. Lamar, and D. Cullen (1996c) Expression of lip genes during growth in soil and oxidation of anthracene by *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **62**: 3697–3703.

Bogan, B.W., R.T. Lamar, W.D. Burgos, and M. Tien (1999) Extent of humification of anthracene, fluoranthene and benzo[*a*]pyrene by *Pleurotus ostreatus* during growth in PAH-contaminated soils. *Lett. Appl. Microbiol.* **28**: 250–254.

Boominathan, K., and C.A. Reddy (1992) cAMP-mediated differential regulation of lignin peroxidase and manganese-dependent peroxidase production in the white-

rot basidiomycete *Phanerochaete chrysosporium*. *Proc. Natl. Acad. Sci. USA* **89**: 5586–5590.

Boonchan, S., M.L. Britz, and G.A. Stanley (2000) Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal–bacterial cocultures. *Appl. Environ. Microbiol.* **66**: 1007–1019.

Bourbonnais, R., D. Leech, and M.G. Paice (1998) Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. *Biochim. Biophys. Acta* **1379**: 381–390.

Boyle, D., C. Wiesner, and A. Richardson (1998) Factors affecting the degradation of polyaromatic hydrocarbons in soil by white-rot fungi. *Soil Biol. Biochem.* **30**: 873–882.

Bressler, D.C., P.M. Fedorak, and M.A. Pickard (2000) Oxidation of carbazole, *N*-ethylcarbazole, fluorene, and dibenzothiophene by the laccase of *Coriolopsis gallica*. *Biotechnol. Lett.* **22**: 1119–1125.

Brodkorb, T.S., and R.L. Legge (1992) Enhanced biodegradation of phenanthrene in oil tar-contaminated soils supplemented with *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **58**: 3117–3121.

Brown, J.A., J.K. Glenn, and M.H. Gold (1990) Manganese regulates expression of manganese peroxidase by *Phanerochaete chrysosporium*. *J. Bacteriol.* **172**: 3125–3130.

Buettner, G.R. (1993) The pecking order of free radicals and antioxidants: lipid peroxidation, α-tocopherol, and ascorbate. *Arch. Biochem. Biophys.* **300**: 535–543.

Bumpus, J.A. (1989) Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **55**: 154–158.

Bumpus, J.A., M. Tien, D. Wright, and S.D. Aust (1985) Oxidation of persistent organic pollutants by a white rot fungus. *Science* **228**: 1434–1436.

Burgos, W.D., J.T. Novak, and D.F. Berry (1996) Reversible sorption and irreversible binding of naphthalene and α-naphthol to soil: elucidation of processes. *Environ. Sci. Technol.* **30**: 1205–1211.

Buswell, J.A. (1994) Potential of spent mushroom substrate for bioremediation purposes. *Compost Sci. Util.* **2**: 31–36.

Cameron, M.D., S. Timofeevski, and S.D. Aust (2000) Enzymology of *Phanerochaete chrysosporium* with respect to the degradation of recalcitrant compounds and xenobiotics. *Appl. Microbiol. Biotechnol.* **54**: 751–758.

Canet, R., J.M. Lopez-Real, and A.J. Beck (1999) Overview of polycyclic aromatic hydrocarbon biodegradation by white-rot fungi. *Land Contam. Reclam.* **7**: 191–197.

Canet, R., J.G. Birnstingl, D.G. Malcolm, J.M. Lopez-Real, and A.J. Beck (2001) Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by native microflora and combinations of white-rot fungi in a coal-tar contaminated soil. *Bioresour. Technol.* **76**: 113–117.

Carmichael, A.B., and L.L. Wong (2001) Protein engineering of *Bacillus megaterium* CYP102: the oxidation of polycyclic aromatic compounds. *Eur. J. Biochem./FEBS* **268**: 3117–3125.

Casillas, R.P., S.A. Crow, Jr., T.M. Heinze, J. Deck, and C.E. Cerniglia (1996) Initial oxidative and subsequent metabolites produced during the metabolism of phenanthrene by fungi. *J. Ind. Microbiol.* **16**: 205–215.

Cerniglia, C.E. (1993) Biodegradation of polycyclic aromatic hydrocarbons. *Curr. Opin. Biotechnol.* **4**: 331–338.

Cerniglia, C.E. (1997) Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. *J. Ind. Microbiol. Biotechnol.* **19**: 324–333.

Cerniglia, C.E. (2003) Recent advances in the biodegradation of polycyclic aromatic hydrocarbons by *Mycobacterium* species. In: *The Utilization of Bioremediation to Reduce Soil Contamination: Problems and Solutions*, V. Sasek, J.A. Glaser, and P. Baveye, eds. Kluwer, Dordrecht, The Netherlands, pp. 51–74.

Cerniglia, C.E., and S.A. Crow (1981) Metabolism of aromatic hydrocarbons by yeasts. *Arch. Microbiol.* **129**: 9–13.

Cerniglia, C.E., and D.T. Gibson (1977) Metabolism of naphthalene by *Cunninghamella elegans*. *Appl. Environ. Microbiol.* **34**: 366–370.

Cerniglia, C.E., and D.T. Gibson (1980a) Oxidation of benzo[*a*]pyrene by the filamentous fungus *Cunninghamella elegans*. *J. Biol. Chem.* **254**: 12174–12180.

Cerniglia, C.E., and D.T. Gibson (1980b) Fungal oxidation of benzo[*a*]pyrene and (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene: evidence for the formation of a benzo[*a*]pyrene 7,8-diol-9,10-epoxide. *J. Biol. Chem.* **255**: 5159–5163.

Cerniglia, C.E., and J.B. Sutherland (2001) Bioremediation of polycyclic aromatic hydrocarbons by ligninolytic and non-ligninolytic fungi. In: *Fungi in Bioremediation*, G.M. Gadd, ed. Cambridge University Press, Cambridge, pp. 136–187.

Cerniglia, C.E., R.L. Hebert, P.J. Szaniszlo, and D.T. Gibson (1978) Fungal transformation of naphthalene. *Arch. Microbiol.* **117**: 135–143.

Cerniglia, C.E., D.T. Gibson, and C. Van Baalen (1980a) Algal oxidation of naphthalene. *J. Gen. Microbiol.* **116**: 495–500.

Cerniglia, C.E., R.H. Dodge, and D.T. Gibson (1980b) Studies on the fungal oxidation of polycyclic aromatic hydrocarbons. *Bot. Mar.* **23**: 121–124.

Cerniglia, C.E., R.H. Dodge, and D.T. Gibson (1982) Fungal oxidation of 3-methylcholanthrene: formation of proximate carcinogenic metabolites of 3-methylcholanthrene. *Chem.-Biol. Interact.* **38**: 161–173.

Cerniglia, C.E., G.L. White, and R.H. Heflich (1985) Fungal metabolism and detoxification of polycycyclic aromatic hydrocarbons. *Arch. Microbiol.* **143**: 105–110.

Cerniglia, C.E., J.B. Sutherland, and S.A. Crow (1992) Fungal metabolism of aromatic hydrocarbons. In: *Microbial Degradation of Natural Products*, G. Winkelmann, ed. VCH, Weinheim, Germany, pp. 193–217.

Cerniglia, C.E., D.T. Gibson, and R.H. Dodge (1994) Metabolism of benz[*a*]anthracene by the filamentous fungus *Cunninghamella elegans*. *Appl. Environ. Microbiol.* **60**: 3931–3938.

Chun, C.L., J.-J. Lee, and J.-W. Park (2002) Solubilization of PAH mixtures by three different anionic surfactants. *Environ. Pollut.* **118**: 307–313.

Collins, P.J., and A.D.W. Dobson (1996) Oxidation of fluorene and phenanthrene by Mn(II) dependent peroxidase activity in whole cultures of *Trametes* (*Coriolus*) *versicolor*. *Biotechnol. Lett.* **18**: 801–804.

Collins, P.J., M.J.J. Kotterman, J.A. Field, and A.D.W. Dobson (1996) Oxidation of anthracene and benzo[*a*]pyrene by laccases from *Trametes versicolor*. *Appl. Environ. Microbiol.* **62**: 4563–4567.

Cutright, T.J. (1995) Polycyclic aromatic hydrocarbon biodegradation and kinetics using *Cunninghamella echinulata* var. *elegans*. *Int. Biodeterior. Biodegrad.* **35**: 397–408.

da Silva, M., C.E. Cerniglia, J.V. Pothuluri, V.P. Canhos, and E. Esposito (2003) Screening filamentous fungi isolated from estuarine sediments for the ability to oxidize polycyclic aromatic hydrocarbons. *World J. Microbiol. Biotechnol.* **19**: 399–405.

da Silva, M., E. Esposito, J.D. Moody, V.P. Canhos, and C.E. Cerniglia (2004) Metabolism of aromatic hydrocarbons by the filamentous fungus *Cyclothyrium* sp. *Chemosphere* **57**: 943–952.

Datta, D., and T.B. Samanta (1988) Effects of inducers of metabolism of benzo[*a*]pyrene in vivo and in vitro: analysis by high pressure liquid chromatography. *Biochem. Biophys. Res. Commun.* **155**: 493–502.

Davis, M.W., J.A. Glaser, J.W. Evans, and R.T. Lamar (1993) Field evaluation of the lignin-degrading fungus *Phanerochaete sordida* to treat creosote-contaminated soil. *Environ. Sci. Technol.* **27**: 2572–2576.

Dawel, G., M. Kastner, J. Michels, W. Poppitz, W. Gunther, and W. Fritsche (1997) Structure of a laccase-mediated product of coupling of 2,4-diamino-6-nitrotoluene to guaiacol, a model for coupling of 2,4,6-trinitrotoluene metabolites to a humic organic soil matrix. *Appl. Environ. Microbiol.* **63**: 2560–2565.

Dombrovskaya, E.N., and S.S. Kostyshin (1996) Effects of surfactants of different ionic nature on the ligninolytic enzyme complexes of the white-rot fungi *Pleurotus floridae* and *Phellinus igniarius*. *Biochemistry* (*Moscow*) **61**: 215–220.

Dosoretz, C.G., and H.E. Grethlein (1991) Physiological aspects of the regulation of extracellular enzymes of *Phanerochaete chrysosporium*. *Appl. Biochem. Biotechnol.* **28–29**: 253–265.

Eggen, T. (1999) Application of fungal substrate from commercial mushroom production—*Pleurotus ostreatus*—for bioremediation of creosote contaminated soil. *Int. Biodeterior. Biodegrad.* **44**: 117–126.

Eggen, T., and A. Majcherczyk (1998) Removal of polycyclic aromatic hydrocarbons (PAH) in contaminated soil by white rot fungus *Pleurotus ostreatus*. *Int. Biodeterior. Biodegrad.* **41**: 111–117.

Eggen, T., and P. Sveum (1999) Decontamination of aged creosote polluted soil: the influence of temperature, white rot fungus *Pleurotus ostreatus*, and pre-treatment. *Int. Biodeterior. Biodegrad.* **43**: 125–133.

Eichlerova, I., L. Homolka, F. Nerud, F. Zadrazil, and P. Baldrian (2000) Screening of *Pleurotus ostreatus* isolates for their ligninolytic properties during cultivation on natural substrates. *Biodegradation* **11**: 279–287.

Field, J.A., E. de Jong, G. Feijoo Costa, and J.A.M. de Bont (1992) Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white-rot fungi. *Appl. Environ. Microbiol.* **58**: 2219–2226.

Field, J.A., E. de Jong, G. Feijoo Costa, and J.A.M. de Bont (1993) Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. *Trends Biotechnol.* **11**: 44–49.

Field, J.A., F. Boelsma, H. Baten, and W.H. Rulkens (1995a) Oxidation of anthracene in water/solvent mixtures by the white-rot fungus *Bjerkandera* sp. strain BOS55. *Appl. Microbiol. Biotechnol.* **44**: 234–240.

Field, J.A., H. Fieken, A. Hage, and M.J.J. Kotterman (1995b) Application of a white-rot fungi to biodegrade benzo[*a*]pyrene in soil. In: *Bioaugmentation for Site Remediation*, R.E. Hinchee, J. Fredrickson, and B.C. Alleman, eds. Battelle Press, Columbus, OH, pp. 165–171.

Field, J.A., H. Baten, F. Boelsma, and W.H. Rulkens (1996a) Biological elimination of polycyclic aromatic hydrocarbons in solvent extracts of polluted soil by the white rot fungus *Bjerkandera* sp. strain BOS55. *Environ. Technol.* **17**: 317–323.

Field, J.A., R.H. Vledder, J.G. van Zelst, and W.H. Rulkens (1996b) The tolerance of lignin peroxidase and manganese-dependent peroxidase to miscible solvents and the in vitro oxidation of anthracene in solvent: water mixtures. *Enzyme Microb. Technol.* **18**: 300–308.

Flowers, L., S.T. Ohnishi, and T.M. Penning (1997) DNA strand scission by polycyclic aromatic hydrocarbon *o*-quinone: role of reactive oxygen species, Cu(II)/Cu(I) redox cycling, and *o*-semiquinone anion radicals. *Biochemistry* **36**: 8640–8648.

Galliano, H., G. Gas, J.I. Seris, and A.M. Boudet (1991) Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes: Mn peroxidase and laccase. *Enzyme Microb. Technol.* **13**: 478–482.

Garon, D., S. Krivobok, and F. Seigle-Murandi (2000) Fungal degradation of fluorene. *Chemosphere* **40**: 91–97.

Garon, D., S. Krivobok, D. Wouessidjewe, and F. Seigle-Murandi (2002) Influence of surfactants on solubilization and fungal degradation of fluorene. *Chemosphere* **47**: 303–309.

Garon, D., L. Sage, D. Wouessidjewe, and F. Seigle-Murandi (2004) Enhanced degradation of fluorene in soil slurry by *Absidia cylindrospora* and maltosyl-cyclodextrin. *Chemosphere* **56**: 159–166.

Gaskell, J., P. Stewart, P.J. Kersten, S.F. Covert, J. Reiser, and D. Cullen (1994) Establishment of genetic linkage by allele-specific polymerase chain reaction: application to the lignin peroxidase gene family of *Phanerochaete chrysosporium*. *Bio/Technol.* **12**: 1372–1375.

Ghosh, D.K., D. Datta, T.B. Samanta, and A.K. Mishra (1983) Microsomal benzo[*a*]pyrene hydroxylase in *Aspergillus ochraceus* TS: assay and characterization of the enzyme system. *Biochem. Biophys. Res. Commun.* **113**: 497–505.

Giraud, F., P. Guiraud, M. Kadri, G. Blake, and R. Steiman (2001) Biodegradation of anthracene and fluoranthene by fungi isolated from an experimental constructed wetland for wastewater treatment. *Water Res.* **35**: 4126–4136.

Glumoff, T., P.J. Harvey, S. Molinari, and M. Goble, et al. (1990) Lignin peroxidase from *Phanerochaete chrysosporium*: molecular and kinetic characterization of isozymes. *Eur. J. Biochem.* **187**: 515–520.

Goodwin, D.C., S.D. Aust, and T.A. Grover (1995) Evidence for veratryl alcohol as a redox mediator in lignin peroxidase-catalyzed oxidation. *Biochemistry* **34**: 5060–5065.

Goyal, A.K., and G.J. Zylstra (1996) Molecular cloning of novel genes for polycyclic aromatic hydrocarbon degradation from *Comamonas testosteroni*. *Appl. Environ. Microbiol.* **62**: 230–236.

Gramss, G., K.D. Voigt, and B. Kirsche (1999) Degradation of polycyclic aromatic hydrocarbons with three to seven aromatic rings by higher fungi in sterile and unsterile soils. *Biodegradation* **10**: 51–62.

Gray, M.R., D.K. Banerjee, P.M. Fedorak, A. Hashimoto, J.H. Masliyah, and M.A. Pickard (1994) Biological remediation of anthracene-contaminated soil in rotating bioreactors. *Appl. Microbiol. Biotechnol.* **40**: 933–940.

Guha, S., C.A. Peters, and P.R. Jaffe (1999) Multisubstrate biodegradation kinetics of naphthalene, phenanthrene, and pyrene mixtures. *Biotechnol. Bioeng.* **65**: 491–499.

Guieysse, B., M.D. Cirne, and B. Mattiasson (2001) Microbial degradation of phenanthrene and pyrene in a two-liquid phase-partitioning bioreactor. *Appl. Microbiol. Biotechnol.* **56**: 796–802.

Haemmerli, S.D., M.S.A Leisola, D. Sanglard, and A. Fiechter (1986) Oxidation of benzo(*a*)pyrene by extracellular ligninases of *Phanerochaete chrysosporium*. *J. Biol. Chem.* **261**: 6900–6903.

Hammel, K.E. (1995) Mechanisms for polycyclic aromatic hydrocarbon degradation by ligninolytic fungi. *Environ. Health Perspect.* **103**: 41–43.

Hammel, K.E., B. Kalyanaraman, and T.K. Kirk (1986) Oxidation of polycyclic aromatic hydrocarbons and dibenzo(*p*)-dioxins by *Phanerochaete chrysosporium* ligninase. *J. Biol. Chem.* **261**: 16948–16952.

Hammel, K.E., B. Green, and W. Zhi-Gai (1991) Ring fission of anthracene by a eucaryote. *Proc. Natl. Acad. Sci. USA* **88**: 10605–10608.

Hammel, K.E., W. Zhi-Gai, B. Green, and M.A. Moen (1992) Oxidative degradation of phenanthrene by the ligninolytic fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **58**: 1832–1838.

Hammel, K.E., K.A. Jensen, M.D. Mozuch, L.L. Landucci, M. Tien, and E.A. Pease (1993) Ligninolysis by a purified peroxidase. *J. Biol. Chem.* **268**: 12274–12281.

Hofmann, K.H. (1986) Oxidation of naphthalene by *Saccharomyces cerevisiae* and *Candida utilis*. *J. Basic Microbiol.* **26**: 109–111.

Holroyd, M.L., and P. Caunt (1994) Fungal processing: a second generation biological treatment for the degradation of recalcitrant organics in soil. *Land Contam. Reclam.* **2**: 183–188.

Huang, X.-D., Y. El-Alawi, D.M. Penrose, B.R. Glick, and B.M. Greenberg (2004) A multi-process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. *Environ. Pollut.* **130**: 465–476.

IARC (International Agency for Research on Cancer) (1983) *Polynuclear Aromatic Compounds*, P. I. World Health Organization, Lyon, France.

in der Wiesche, C., R. Martens, and F. Zadrazil (1996) Two-step degradation of pyrene by white-rot fungi and soil microorganisms. *Appl. Microbiol. Biotechnol.* **46**: 653–659.

Jeftic, L., and R.N. Adams (1970) Electrochemical oxidation pathways of benzo[*a*]pyrene. *J. Am. Chem. Soc.* **92**: 1332–1337.

Jensen, K.A., Jr., K.M. Evans, T.K. Kirk, and K.E. Hammel (1994) Biosynthetic pathway for veratryl alcohol in the ligninolytic fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **60**: 709–714.

Johannes, C., A. Majcherczyk, and A. Huttermann (1996) Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediator compounds. *Appl. Microbiol. Biotechnol.* **46**: 313–317.

Johannes, C., A. Majcherczyk, and A. Huttermann (1998) Oxidation of acenaphthene and acenaphthylene by laccase of *Trametes versicolor* in a laccase-mediator system. *J. Biotechnol.* **61**: 151–156.

Johansson, T.K., and P.O. Nyman (1996) A cluster of genes encoding major isozymes of lignin peroxidase and manganese peroxidase from the white rot fungus *Trametes versicolor. Gene* **170**: 31–38.

Juhasz, A.L., and R. Naidu (2000) Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[*a*]pyrene. *Int. Biodeterior. Biodegrad.* **45**: 57–88.

Kaal, E.E.J., E. de Jong, and J.A. Field (1993) Stimulation of ligninolytic peroxidase activity by nitrogen nutrients in the white rot fungus *Bjerkandera* sp. strain BOS55. *Appl. Environ. Microbiol.* **59**: 4031–4036.

Kamely, D., A. Chakrabarty, and G.S. Emenn, eds. (1990) *Biotechnology and Biodegradation*. Gulf Publishing Company, Houston, TX.

Kapich, A., M. Hofrichter, T. Vares, and A. Hatakka (1999) Coupling of manganese peroxidase-mediated lipid peroxidation with destruction of nonphenolic lignin model compounds and ^{14}C-labeled lignins. *Biochem. Biophys. Res. Commun.* **259**: 212–219.

Kapoor, M., and W.S. Lin (1984) Studies on the induction of aryl hydrocarbon hydroxylase in *Neurospora crassa* and its suppression by sodium selenite. *Xenobiotica* **14**: 903–915.

Keith, L.H., and W.A. Telliard (1979) Priority pollutants. I. A perspective view. *Environ. Sci. Technol.* **13**: 416–423.

Kellner, D.G., S.A. Maves, and G. Sligar (1997) Engineering cytochrome P450s for bioremediation. *Curr. Opin. Biotechnol.* **8**: 274–278.

Kennes, C., and J.M. Lema (1994) Degradation of major compounds of creosotes (PAH and phenols) by *Phanerochaete chrysosporium. Biotechnol. Lett.* **16**: 759–764.

Kiehlmann, E., L. Pinto, and M. Moore (1996) The biotransformation of chrysene to *trans*-1,2-dihydroxy-1,2-dihydrochrysene by filamentous fungi. *Can. J. Microbiol.* **42**: 604–608.

Kim, J.-E., E. Fernandes, and J.-M. Bollag (1997) Enzymatic coupling of the herbicide bentazon with humus monomers and characterization of reaction products. *Environ. Sci. Technol.* **31**: 2392–2398.

Kotterman, M.J.J., E. Heessels, E. de Jong, and J.A. Field (1994) The physiology of anthracene biodegradation by the white-rot fungus *Bjerkandera* sp. strain BOS55. *Appl. Microbiol. Biotechnol.* **42**: 179–186.

Kotterman, M.J.J., R.A. Wasseveld, and J.A. Field (1996) Hydrogen peroxide production as a limiting factor in xenobiotic compound oxidation by nitrogen-sufficient cultures of *Bjerkandera* sp. strain BOS55 overproducing peroxidases. *Appl. Environ. Microbiol.* **62**: 880–885.

Kotterman, M.J.J., E.H. Vis, and J.A. Field (1998a) Polycyclic aromatic hydrocarbon oxidation by the white-rot fungus *Bjerkandera* sp. strain BOS55 in the presence of nonionic surfactants. *Biotechnol. Bioeng.* **57**: 220–227.

Kotterman, M.J.J., E.H. Vis, and J.A. Field (1998b) Successive mineralization and detoxification of benzo[*a*]pyrene by the white rot fungus *Bjerkandera* sp. strain BOS55 and indigenous microflora. *Appl. Environ. Microbiol.* **64**: 2853–2858.

Kremer, S., and H. Anke (1997) Fungi in bioremediation. In: *Fungal Biotechnology*, T. Anke, ed. Chapman & Hall, Weinheim, Germany, pp. 275–295.

Krivobok, S., E. Miriouchkine, F. Seigle-Murandi, and J.-L. Benoit-Guyod (1998) Biodegradation of anthracene by soil fungi. *Chemosphere* **37**: 523–530.

Kullman, S.W., and F. Matsumura (1997) Identification of a novel cytochrome P-450 gene from the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **63**: 2741–2746.

Kurtzmann, C.P., and J.W. Fell (1998) *The Yeasts: A Taxonomic Study*, 4th ed. Elsevier, Amsterdam, The Netherlands.

Lamar, R.T., and J.A. Glaser (1994) Field evaluation of the remediation of soils contaminated with wood-preserving chemicals using lignin-degrading fungi. In: *Bioremediation of Chlorinated and Polycyclic Aromatic Compounds*, R.E. Hinchee, A. Leeson, L. Semprini, and S.K. Ong., eds. CRC Press, Boca Raton, FL, pp. 203–217.

Lamar, R.T., B. Schoenike, A.Vanden Wymelenberg, P. Stewart, et al. (1995) Quantitation of fungal mRNA in complex substrates by reverse transcription PCR and its application to *Phanerochaete chrysosporium*-colonized soil. *Appl. Environ. Microbiol.* **61**: 2122–2126.

Lambert, M., S. Kremer, O. Sterner, and H. Anke (1994) Metabolism of pyrene by the basidiomycete *Crinipellis stipitaria* and identification of pyrenequinones and their hydroxylated precursors in strain JK375. *Appl. Environ. Microbiol.* **60**: 3597–3601.

Lambert, M., S. Kremer, and H. Anke (1995) Antimicrobial, phytotoxic, nematicidal, cytotoxic, and mutagenic activities of 1-hydroxypyrene, the initial metabolite in pyrene metabolism by the basidiomycete *Crinipellis stipitaria*. *Bull. Environ. Contam. Toxicol.* **55**: 251–257.

Lange, B., S. Kremer, O. Sterner, and H. Anke (1994) Pyrene metabolism in *Crinipellis stipitaria*: identification of *trans*-4,5-dihydro-4,5-dihydroxypyrene and 1-pyrenylsulfate in strain JK364. *Appl. Environ. Microbiol.* **60**: 3602–3607.

Lange, B., S. Kremer, O. Sterner, and H. Anke (1996) Metabolism of pyrene by basidiomycetous fungi of the genera *Crinipellis*, *Marasmius*, and *Marasmiellus*. *Can. J. Microbiol.* **42**: 1179–1183.

Lassen, P., A. Randall, O. Jorgensen, P. Warwick, and L. Carlsen (1994) Enzymatically mediated incorporation of 2-chlorophenol and 4-chlorophenol into humic acids. *Chemosphere* **28**: 703–710.

Launen, L.A., L.J. Pinto, C. Wiebe, E. Kiehlmann, and M.M. Moore (1995) The oxidation of pyrene and benzo[*a*]pyrene by nonbasidiomycete soil fungi. *Can. J. Microbiol.* **41**: 477–488.

Launen, L.A., L.J. Pinto, and M.M. Moore (1999) Optimization of pyrene oxidation by *Penicillium janthinellum* using response-surface methodology. *Appl. Microbiol. Biotechnol.* **51**: 510–515.

Launen, L.A., L.J. Pinto, P.W. Percival, S.F.S. Lam, and M.M. Moore (2000) Pyrene is metabolized to bound residues by *Penicillium janthinellum* SFU403. *Biodegradation* **11**: 305–312.

Leontievsky, A.A., T. Vares, P. Lankinen, J.K. Shergill, et al. (1997) Blue and yellow laccases of lignolytic fungi. *FEMS Microbiol. Lett.* **156**: 9–14.

Levin, L., A. Viale, and A. Forchiassin (2003) Degradation of organic pollutants by the white rot basidiomycete *Trametes trogii*. *Int. Biodeterior. Biodegrad.* **52**: 1–5.

Liao, W.-L., and D.-H. Tseng (1996) Biotreatment of naphthalene by PAH-acclimated pure culture with white-rot fungus *Phanerochaete chrysosporium*. *Water Sci. Technol.* **34**: 73–79.

Liao, W.-L., D.-H. Tseng, Y.-C. Tsai, and S.-C. Chang (1997) Microbial removal of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. *Water Sci. Technol.* **35**: 254–264.

Lisowska, K., and J. Dlugonski (1999) Removal of anthracene and phenanthrene by filamentous fungi capable of cortexolone 11-hydroxylation. *J. Basic Microbiol.* **39**: 117–125.

Liste, H.H., and M. Alexander (2000) Plant-promoted pyrene degradation in soil. *Chemosphere* **40**: 7–10.

Lobos, S., L. Larrondo, L. Sales, E. Karahanian, and R. Vicuna (1994) Isoenzymes of manganese-dependent peroxidase and laccase produced by the lignin degrading basidiomycete *Ceriporiopsis subvermispora*. *Microbiology* **140**: 2691–2698.

MacGillivray, R.A., and M.P. Shiaris (1993) Biotransformation of polycyclic aromatic hydrocarbons by yeasts isolated from coastal sediments. *Appl. Environ. Microbiol.* **59**: 1613–1618.

Mahro, B., G. Schaefer, and M. Kastner (1994) Pathways of microbial degradation of polycyclic aromatic hydrocarbons in soil. In: *Bioremediation of Chlorinated and Polycyclic Aromatic Compounds*, R.E. Hinchee, A. Leeson, L. Semprini, and S.K. Ong, eds. CRC Press, Boca Raton, FL, pp. 203–217.

Majcherczyk, A., and A. Huttermann (1993) Behandlung mit Weißfaulepilzen als Weg zur Rezyklisierung von Altholz. In: Die pflanzliche Zellwand als Vorbild fur Holzwerkstoffe, A. Huttermann and R. Kharazipour, eds. Sauerlanders Verlag, Frankfurt, Germany, pp. 69–82.

Majcherczyk, A., and C. Johannes (2000) Radical mediated indirect oxidation of a PEG-coupled polycyclic aromatic hydrocarbon (PAH) model compound by fungal laccase. *Biochim. Biophys. Acta* **1474**: 157–162.

Majcherczyk, A., C. Johannes, and A. Huttermann (1998) Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. *Enzyme Microb. Technol.* **22**: 335–341.

Majcherczyk, A., C. Johannes, and A. Huttermann (1999) Oxidation of aromatic alcohols by laccase from *Trametes versicolor* mediated by the 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) cation radical and dication. *Appl. Microbiol. Biotechnol.* **51**: 267–276.

Male, B., R. Male, S. Brown, and J.H.T. Luong (1995) Enzymatic oxidation of water-soluble cyclodextrin–polynuclear aromatic hydrocarbon inclusion complexes, using lignin peroxidase. *Enzyme Microb. Technol.* **17**: 607–614.

Maltseva, O.V., M.-L. Niku-Paavola, A.A. Leontievsky, N.M. Myasoedova, and L.A. Golovleva (1991) Ligninolytic enzymes of the white rot fungus *Panus tigrinus*. *Biotechnol. Appl. Biochem.* **13**: 291–302.

Martens, R., and F. Zadrazil (1998) Screening of white-rot fungi for their ability to mineralize polycyclic aromatic hydrocarbons in soil. *Folia Microbiol.* **43**: 97–103.

Martens, R., M. Wolter, M. Bahadir, and F. Zadrazil (1999) Mineralization of ^{14}C-labelled highly-condensed polycyclic aromatic hydrocarbons in soil by *Pleurotus* sp. Florida. *Soil Biol. Biochem.* **31**: 1893–1899.

Masaphy, S., D. Levanon, Y. Henis, K. Venkateswarlu, and S.L. Kelly (1995) Microsomal and cytosolic cytochrome P450-mediated benzo[*a*]pyrene hydroxylation in *Pleurotus pulmonarius. Biotechnol. Lett.* **17**: 969–974.

Masaphy, S., D. Levanon, Y. Henis, K. Venkateswarlu, and S.L. Kelly (1996) Evidence for cytochrome P-450 and P-450 mediated benzo[*a*]pyrene hydroxylation in the white rot fungus *Phanerochaete chrysosporium. FEMS Microbiol. Lett.* **135**: 51–55.

May, R., P. Schroder, and H. Sandermann, Jr. (1997) Ex-situ process for treating PAH-contaminated soil with *Phanerochaete chrysosporium. Environ. Sci. Technol.* **31**: 2626–2633.

Mayer, A.M., and R.C. Staples (2002) Laccase: new functions for an old enzyme. *Phytochemistry* **60**: 551–565.

McFarland, M.J., and X.J. Qiu (1995) Removal of benzo(*a*)pyrene in soil composting systems amended with the white rot fungus *Phanerochaete chrysosporium. J. Hazard. Mater.* **42**: 61–70.

McFarland, M.J., X.J. Qiu, J.L. Sims, M.E. Randolph, and R.C. Sims (1992) Remediation of petroleum impacted soils in fungal compost bioreactors. *Water Sci. Technol.* **25**: 197–206.

McMillan, D.C., P.P. Fu, J.P. Freeman, D.W. Miller, and C.E. Cerniglia (1988) Microbial metabolism and detoxification of 7,12-dimethylbenz[*a*]anthracene. *J. Ind. Microbiol.* **3**: 211–225.

Meador, J.P., J.E. Stein, W.L. Reichert, and U. Varanasi (1995) Bioaccumulation of polycyclic aromatic hydrocarbons by marine organisms. *Rev. Environ. Contam. Toxicol.* **143**: 79–163.

Menn, F.M., J.P. Easter, and G.S. Sayler (2000) Genetically engineered microorganisms and bioremediation. In: *Biotechnology*, Vol. IIb, *Environmental Processes II*, H.-J. Rehm and G. Reed, eds. Wiley, New York, pp. 442–463.

Messerschmidt, A. (1993) Blue copper oxidases. *Adv. Inorg. Chem.* **40**: 121–185.

Messerschmidt, A., ed. (1997) *Multi-copper Oxidases*. World Scientific, Singapore.

Mester, T., and J.A. Field (1998) Characterization of a novel manganese peroxidase-lignin peroxidase hybrid enzyme produced by *Bjerkandera* sp. strain BOS55 in the absence of manganese. *J. Biol. Chem.* **273**: 15412–15417.

Mester, T., and M. Tien (2000) Oxidation mechanism of ligninolytic enzymes involved in the degradation of environmental pollutants. *Int. Biodeterior. Biodegrad.* **46**: 51–59.

Millis, C.D., D. Cai, M.T. Stankovich, and M. Tien (1989) Oxidation-reduction potentials and ionization states of extracellular peroxidases from the lignin-degrading fungus *Phanerochaete chrysosporium. Biochemistry* **28**: 8484–8489.

Moen, M.A., and K.E. Hammel (1994) Lipid peroxidation by the manganese peroxidase by *Phanerochaete chrysosporium* is the basis of phenanthrene oxidation by the intact fungus. *Appl. Environ. Microbiol.* **60**: 1956–1961.

Mollea, C., F. Bosco, and B. Ruggeri (2005) Fungal biodegradation of naphthalene: microcosm studies. *Chemosphere* **60**: 636–643.

Morgan, P., S.A. Lee, S.T. Lewis, A.N. Sheppard, and R.J. Watkinson (1993) Growth and biodegradation by white-rot fungi inoculated into soil. *Soil Biol. Biochem*. **25**: 279–287.

Moukha, S.M., H.A.B. Wosten, E.-J. Mylius, M. Asther, and J.G.H. Wessels (1993) Spatial and temporal accumulation of mRNAs encoding two common lignin per-oxidases in *Phanerochaete chrysosporium*. *J. Bacteriol*. **175**: 3672–3678.

Munoz, C., F. Guillen, A.T. Martinez, and M.J. Martinez (1997) Laccase isoenzymes of *Pleurotus eryngii*: characterization, catalytic properties, and participation in activation of molecular oxygen and Mn^{2+} oxidation. *Appl. Environ. Microbiol*. **63**: 2166–2174.

Narro, M.L., C.E. Cerniglia, C. Van Baalen, and D.T. Gibson (1992a) Metabolism of phenanthrene by the cyanobacterium *Agmenellum quadruplicatum* PR-6. *Appl. Environ. Microbiol*. **58**: 1351–1359.

Narro, M.L., C.E. Cerniglia, C. Van Baalen, and D.T. Gibson (1992b) Evidence of NIH shift in naphthalene oxidation by the marine cyanobacterium, *Oscillatoria* species strain JCM. *Appl. Environ. Microbiol*. **58**: 1360–1363.

Nedunuri, K.V., R.S. Govindaraju, M.K. Banks, A.P. Schwab, and Z. Chen (2000) Evaluation of phytoremediation for field-scale degradation of total petroleum hydrocarbons. *J. Environ. Eng*. **126**: 483–490.

Nerud, F., Z. Zouchova, and Z. Misurcova (1991) Ligninolytic properties of different white-rot fungi. *Biotechnol. Lett*. **13**: 657–660.

Nie, G., N.S. Reading, and S.D. Aust (1999) Relative stability of recombinant versus native peroxidases from *Phanerochaete chrysosporium*. *Arch. Biochem. Biophys*. **365**: 328–334.

Novotny, C., P. Erbanova, V. Sasek, A. Kubatova, T. Cajthaml, E. Lang, J. Krahl, and F. Zadrazil (1999) Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi. *Biodegradation* **10**: 159–168.

Novotny, C., P. Erbanova, T. Cajthaml, N. Rothchild, C. Dosoretz, and V. Sasek (2000) *Irpex lacteus*, a white rot fungus applicable to water and soil bioremedia-tion. *Appl. Microbiol. Biotechnol*. **54**: 850–853.

Omura, T. (1999) Forty years of cytochrome P450. *Biochem. Biophys. Res. Commun*. **266**: 690–698.

Paszczynski, A., V.-B. Huynh, and R.L. Crawford (1985) Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochaete chrysospo-rium*. *FEMS Microbiol. Lett*. **9**: 37–41.

Pickard, M.A., R. Roman, R. Tinoco, and R. Vazquez-Duhalt (1999) Polycyclic aro-matic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. *Appl. Environ. Microbiol*. **65**: 3805–3809.

Pothuluri, J.V., and C.E. Cerniglia (1998) Current aspects of polycyclic aromatic hydrocarbon biodegradation processes. In: *Bioremediation: Principles and Prac-tice—Biodegradation Technology Developments*, S.K. Sikdar and R.L. Irvine, eds. Technomic Publishing Company, Lancaster, PA, Vol. II, pp. 461–520.

Pothuluri, J.V., J.P. Freeman, F.E. Evans, and C.E. Cerniglia (1990) Fungal transfor-mation of fluoranthene. *Appl. Environ. Microbiol*. **56**: 2974–2983.

Pothuluri, J.V., R.H. Heflich, P.P. Fu, and C.E. Cerniglia (1992a) Fungal metabolism and detoxification of fluoranthene. *Appl. Environ. Microbiol*. **58**: 937–941.

Pothuluri, J.V., J.P. Freeman, F.E. Evans, and C.E. Cerniglia (1992b) Fungal metabolism of acenaphthene by *Cunninghamella elegans*. *Appl. Environ. Microbiol.* **58**: 3654–3659.

Pothuluri, J.V., J.P. Freeman, F.E. Evans, and C.E. Cerniglia (1993) Biotransformation of fluorene by the fungus *Cunninghamella elegans*. *Appl. Environ. Microbiol.* **59**: 1977–1980.

Pothuluri, J.V., A. Selby, F.E. Evans, J.P. Freeman, and C.E. Cerniglia (1995) Transformation of chrysene and other polycyclic aromatic hydrocarbon mixtures by the fungus *Cunninghamella elegans*. *Can. J. Bot.* **73**(Suppl. 1): S1025–S1033.

Pothuluri, J.V., F.E. Evans, T.M. Heinze, P.P. Fu, and C.E. Cerniglia (1996a) Fungal metabolism of 2-nitrofluorene. *J. Toxicol. Environ. Health* **47**: 587–599.

Pothuluri, J.V., F.E. Evans, T.M. Heinze, and C.E. Cerniglia (1996b) Formation of sulfate and glucoside conjugates of benzo[*e*]pyrene by *Cunninghamella elegans*. *Appl. Microbiol. Biotechnol.* **45**: 677–683.

Potin, O., C. Rafin, and E. Veignie (2004a) Bioremediation of an aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil by filamentous fungi isolated from the soil. *Int. Biodeterior. Biodegrad.* **54**: 45–52.

Potin, O., E. Veignie, and C. Rafin (2004b) Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by *Cladosporium sphaerospermum* isolated from an aged PAH contaminated soil. *FEMS Microbiol. Ecol.* **51**: 71–78.

Qiu, X.J., and M.J. McFarland (1991) Bound residue formation in PAH contaminated soil composting using *Phanerochaete chrysosporium*. *Wastes Hazard. Mater.* **8**: 115–126.

Rama, R., C. Mougin, F.-D. Boyer, A. Kollmann, C. Malosse, and J.-C. Sigoillet (1998) Biotransformation of benzo[*a*]pyrene in bench scale reactor using laccase of *Pycnosporus cinnabarinus*. *Biotechnol. Lett.* **20**: 1101–1104.

Randall, D.W., S.D. George, P.L. Holland, B. Hedman, et al. (2001) Spectroscopic and electronic studies of blue copper model complexes. 2. Comparison of three- and four-coordinate Cu(II)-thiolate complexes and fungal laccase. *J. Am. Chem. Soc.* **122**: 11632–11648.

Ravelet, C., S. Krivobok, L. Sage, and R. Steiman (2000) Biodegradation of pyrene by sediment fungi. *Chemosphere* **40**: 557–563.

Ravelet, C., C. Grosset, S. Krivobok, B. Montuelle, and J. Alary (2001) Pyrene degradation by two fungi in a freshwater sediment and evaluation of fungal biomass by ergosterol content. *Appl. Microbiol. Biotechnol.* **56**: 803–808.

Rebhun, M., F. De Smedt, and J. Rwetabula (1996) Dissolved humic substances for remediation of sites contaminated by organic pollutants: binding-desorption model predictions. *Water Res.* **30**: 2027–2038.

Rehmann, K., H.P. Noll, C.E. Steinberg, and A.A. Kettrup (1998) Pyrene biodegradation by *Mycobacterium* sp. strain KR2. *Chemosphere* **36**: 2977–2992.

Rehmann, K., N. Hertkorn, and A.A. Kettrup (2001) Fluoranthene metabolism in *Mycobacterium* sp. strain KR20: identity of pathway intermediates during degradation and growth. *Microbiology* **147**: 2783–2794.

Reinert, G., E. Schuster, M. Schneider, and D. Bryniok (1996) Biological cleaning of soil wash dredging: new perspectives by a procedure innovation. *TerraTech* **3**: 55–57.

Rodriguez, E., O. Nuero, F. Guillen, A.T. Martinez, and M.J. Martinez (2004) Degradation of phenolic and non-phenolic aromatic pollutants by four *Pleurotus* species: the role of laccase and versatile peroxidase. *Soil Biol. Biochem.* **36**: 909–916.

Romero, M.C., M.C. Cazau, S. Giorgieri, and A.M. Arambarri (1998) Phenanthrene degradation by microorganisms isolated from a contaminated stream. *Environ. Pollut.* **101**: 355–359.

Romero, M.C., M.L. Salvioli, M.C. Cazau, and A.M. Arambarri (2002) Pyrene degradation by yeasts and filamentous fungi. *Environ. Pollut.* **117**: 159–163.

Ruiz-Duenas, F.J., S. Camarero, M. Perez-Boada, M.J. Martinez, and A.T. Martinez (2001) A new versatile peroxidase from *Pleurotus*. *Biochem. Soc. Trans.* **29**: 116–122.

Ruttimann, C., E. Schwember, L. Salas, D. Cullen, and R. Vicuna (1992) Ligninolytic enzymes of the white rot basidiomycetes *Phlebia brevispora* and *Ceriporiopsis subvermispora*. *Biotechnol. Appl. Biochem.* **16**: 64–76.

Ruttimann-Johnson, C., and R.T. Lamar (1996) Polymerization of pentachlorophenol and ferulic acid by fungal extracellular lignin-degrading enzymes. *Appl. Environ. Microbiol.* **62**: 3890–3893.

Ruttimann-Johnson, C., D. Cullen, and R.T. Lamar (1994) Manganese peroxidases of the white rot fungus *Phanerochaete sordida*. *Appl. Environ. Microbiol.* **60**: 599–605.

Sack, U., and W. Fritsche (1997) Enhancement of pyrene mineralization in soil by wood-decaying fungi. *FEMS Microbiol. Ecol.* **22**: 77–83.

Sack, U., and T. Gunther (1993) Metabolism of PAH by fungi and correlation with extracellular enzymatic activities. *J. Basic Microbiol.* **33**: 269–277.

Sack, U., T.M. Heinze, J. Deck, C.E. Cerniglia, M.C. Cazau, and W. Fritsche (1997a) Novel metabolites in phenanthrene and pyrene transformation by *Aspergillus niger*. *Appl. Environ. Microbiol.* **63**: 2906–2909.

Sack, U., T.M. Heinze, J. Deck, C.E. Cerniglia, R. Martens, F. Zadrazil, and W. Fritsche (1997b) Comparison of phenanthrene and pyrene degradation by different wood-decaying fungi. *Appl. Environ. Microbiol.* **63**: 3919–3925.

Sack, U., M. Hofrichter, and W. Fritsche (1997c) Degradation of polycyclic aromatic hydrocarbons by manganese peroxidase of *Nematoloma frowardii*. *FEMS Microbiol. Lett.* **152**: 227–234.

Salicis, F., S. Krivobok, M. Jack, and J.-L. Benoit-Guyod (1999) Biodegradation of fluoranthene by soil fungi. *Chemosphere* **38**: 3031–3039.

Saraswathy, A., and R. Hallberg (2002) Degradation of pyrene by indigenous fungi from a former gasworks site. *FEMS Microbiol. Lett.* **210**: 227–232.

Saraswathy, A., and R. Hallberg (2005) Mycelia pellet formation by *Penicillium ochrochloron* species due to exposure to pyrene. *Microbiol. Res.* **160**: 375–383.

Sarkar, J.M., R.L. Malcolm, and J.-M. Bollag (1988) Enzymatic coupling of 2,4-dichlorophenol to stream fulvic acid in the presence of oxidoreductases. *Soil Sci. Soc. Am. J.* **52**: 688–694.

Sayler, G.S., and S. Ripp (2000) Field applications of genetically engineered microorganisms for bioremediation processes. *Curr. Opin. Biotechnol.* **11**: 286–289.

Sayler, G.S., U. Matrubutham, F.M. Menn, W.H. Johnston, and R.D. Stapleton (1998) Molecular probes and biosensors in bioremediation and site assessment. In: *Bioremediation: Principles and Practice*, S.K. Sikdar, R.L. Irwine, and P.N. Lancaster, eds. Technomic Publishing Company, Lancaster, PA, Vol. I, pp. 385–434.

Schneider, J., R. Grosser, K. Jayasimhulu, W. Xue, and D. Warshawsky (1996) Degradation of pyrene, benz[*a*]anthracene, and benzo[*a*]pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. *Appl. Environ. Microbiol.* **62**: 13–19.

Schuster, E., and M. Blank-Huber (1994) Soil laundry and biology under a roof. *TerraTech* **1**: 42–45.

Schutzendubel, A., A. Majcherczyk, C. Johannes, and A. Huttermann (1999) Degradation of fluorene, anthracene, phenanthrene, fluoranthene, and pyrene lacks connection to the production of extracellular enzymes by *Pleurotus ostreatus* and *Bjerkandera adusta. Int. Biodeterior. Biodegrad.* **43**: 93–100.

Scott, S.L., W.J. Chen, A. Bakac, and J.H. Espenson (1993) Spectroscopic parameters, electrode potentials, acid ionization constants, and electron exchange rate of the 2,2-azinobis(3-ethylbenzothiazolone-6-sulfonate) radicals and ions. *J. Phys. Chem.* **97**: 6710–6714.

Siegrist, R.L. (1990) Development and implementation of soil quality and clean up criteria for contaminated sites. In: *Contaminated Soil*, K. Wolf, J. van den Brink, and F.J. Colon, eds. Kluwer, Dordrecht, The Netherlands, pp. 149–156.

Sims, P., and P.L. Grover (1981) Involvement of dihydrodiols and diol-epoxides in the metabolic activation of polycyclic aromatic hydrocarbons other than benzo[*a*]pyrene. In: *Polycyclic Hydrocarbons and Cancer*, H.V. Gelboin and P.O.P. Ts'o, eds. Academic Press, New York, Vol. 3, pp. 117–121.

Sinclair, R., B. Copeland, I. Yamazaki, and L. Powers (1995) X-ray absorption spectroscopy comparison of the active site structure of *Phanerochaete chrysosporium* lignin peroxidase isoenzymes H2, H3, H4, H5, H8 and H10. *Biochemistry* **34**: 13176–13182.

Solomon, E.I., U.M. Sundaram, and T.E. Machonkin (1996) Multicopper oxidases and oxygenases. *Chem. Rev.* **96**: 2563–2605.

Solomon, E.I., A.E. Palmer, U.M. Sundaram, and T.E. Machonkin (1998) Spectroscopic studies of O_2 intermediates in copper proteins: electronic structure contributions to function. In: *Bioorganic Chemistry: Spectroscopic Methods in Bioinorganic Chemistry*, E.I. Solomon and K.O. Hodgson, eds. ACS Symposium Series 692. American Chemical Society, Washington, DC, pp. 423–452.

Song, H.G. (1997) Biodegradation of aromatic hydrocarbons by several white-rot fungi. *J. Microbiol.* **35**: 66–71.

Stanley, G.A., M. Britz, S. Boonchan, and A.L. Juhasz (1999) Detoxification of soils containing high molecular weight polycyclic aromatic hydrocarbons by gram-negative bacteria and bacterial–fungal co-cultures. In: *Remediation of Hazardous Waste Contaminated Soil*, D.L. Wise, D.L. Trantolo, E.J. Cichon, H.I. Inyang, and U. Stottmeister, eds. Marcel Dekker, New York, pp. 161–179.

Stewart, P., P. Kersten, A.Vanden Wymelenberg, J. Gaskell, and D. Cullen (1992) Lignin peroxidase gene family of *Phanerochaete chrysosporium*: complex regulation by carbon and nitrogen limitation and identification of a second dimorphic chromosome. *J. Bacteriol.* **174**: 5036–5042.

Story, S.P., S.H. Parker, J.D. Kline, T.R. Tzeng, J.G. Mueller, and E.L. Kline (2000) Identification of four structural genes and two putative promoters necessary for utilization of naphthalene, phenanthrene, fluoranthene by *Sphingomonas paucimobilis* var. EPA505. *Gene* **260**: 155–169.

Surovtseva, E.G., V.S. Ivoilov, and S.S. Beliaev (1999) Physiologo-biochemical properties of a strain of *Beijerinckia mobilis* 1 phi Phn+—a degrader of polycyclic aromatic hydrocarbons. *Mikrobiologiya* **68**: 845–850.

Sutherland, J.B., A.L. Selby, J.P. Freeman, F.E. Evans, and C.E. Cerniglia (1991) Metabolism of phenanthrene by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **57**: 3310–3316.

Sutherland, J.B., A.L. Selby, J.P. Freeman, P.P. Fu, D.W. Miller, and C.E. Cerniglia (1992) Identification of xyloside conjugates formed from anthracene by *Rhizoctonia solani*. *Mycol. Res.* **96**: 509–517.

Sutherland, J.B., F. Raff, A.A. Khan, and C.E. Cerniglia (1995) Mechanisms of polycyclic aromatic hydrocarbon degradation. In: *Microbial Transformation and Degradation of Toxic Organic Chemicals*, L.Y. Young and C.E. Cerniglia, eds. Wiley-Liss, New York, pp. 269–306.

Thurston, C.F. (1994) Structure and function of fungal laccases. *Microbiology* **140**: 19–26.

Tucker, B., C. Radtke, S.-I. Kwon, and A.J. Anderson (1995) Suppression of bioremediation by *Phanerochaete chrysosporium* by soil factors. *J. Hazard. Mater.* **41**: 251–265.

USEPA (1987) *Health Effects Assessment for Acenaphthene*. EPA/600/8-88/010. Environmental Criteria and Assessment Office, U.S. Environmental Protection Agency, Cincinatti, OH.

van den Brink, H(J).M., R.F.M van Gorcom, C.A.M.J.J. van den Hondel, and P.J. Punt (1998) Cytochrome P450 enzyme systems in fungi. *Fungal Genet. Biol.* **23**: 1–17.

Vazquez-Duhalt, R., D.W.S. Westlake, and P.M. Fedorak (1994) Lignin peroxidase oxidation of aromatic compounds in systems containing organic solvents. *Appl. Environ. Microbiol.* **60**: 459–466.

Vazquez-Duhalt, R., M. Ayala, and F.J. Marquez-Rocha (2001) Biocatalytic chlorination of aromatic hydrocarbons by chloroperoxidase of *Caldariomyces fumago*. *Phytochemistry* **58**: 929–933.

Veignie, E., C. Rafin, P. Woisel, A. Lounes-Hadj Sahraoui, and F. Cazier (2002) Metabolization of the polycyclic aromatic hydrocarbon benzo[*a*]pyrene by a non-white rot fungus (*Fusarium solani*) in a batch reactor. *Polycycl. Aromat. Compounds* **22**: 87–97.

Veignie, E., C. Rafin, P. Woisel, and F. Cazier (2004) Preliminary evidence of the role of hydrogen peroxide in the degradation of benzo[*a*]pyrene by a non-white rot fungus *Fusarium solani*. *Environ. Pollut.* **129**: 1–4.

Verdin, A., A. Lounes-Hadj Sahraoui, and R. Durand (2004) Degradation of benzo[a]pyrene by mitosporic fungi and extracellular oxidative enzymes. *Int. Biodeterior. Biodegrad.* **53**: 65–70.

Verdin, A., A. Lounes-Hadj Sahraoui, R. Newsam, G. Robinson, and R. Durand (2005) Polycyclic aromatic hydrocarbons storage by *Fusarium solani* in intracellular lipid vesicles. *Environ. Pollut.* **133**: 283–291.

Vila, J., Z. Lopez, J. Sabate, C. Minguilon, A.M. Solanas, and M. Grifoll (2001) Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. strain AP1: actions of the isolate on two- and three-ring polycyclic aromatic hydrocarbons. *Appl. Environ. Microbiol.* **67**: 5497–5505.

Vyas, B.R.M., S. Bakowski, V. Sasek, and M. Matucha (1994) Degradation of anthracene by selected white rot fungi. *FEMS Microbiol. Ecol.* **14**: 65–70.

Warshawsky, D., T. Cody, M.J. Radike, R. Reilman, B. Schumann, et al. (1995) Biotransformation of benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons and heterocyclic analogs by several green algae and other algal species under gold and white light. *Chem.-Biol. Interact.* **97**: 131–148.

Wilson, S.C., and K.C. Jones (1993) Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. *Environ. Pollut.* **81**: 229–249.

Wolter, M., F. Zadrazil, R. Martens, and M. Bahadir (1997) Degradation of eight highly condensed polycyclic aromatic hydrocarbons by *Pleurotus* sp. Florida in solid wheat straw substrate. *Appl. Microbiol. Biotechnol.* **48**: 398–404.

Wong, L.K., J. Dru, L.-S. Lin, and J. Knapp (1983) Metabolism of 7,12-dimethylbenz-[*a*]anthracene by *Cunninghamella elegans. Appl. Environ. Microbiol.* **46**: 1239–1242.

Wunch, K.G., T. Feibelman, and J.W. Bennett (1997) Screening of fungi capable of removing benzo[a]pyrene in culture. *Appl. Microbiol. Biotechnol.* **47**: 620–624.

Wunder, T., S. Kremer, O. Sterner, and H. Anke (1994) Metabolism of the polycyclic aromatic hydrocarbon pyrene by *Aspergillus niger* SK 9317. *Appl. Microbiol. Biotechnol.* **42**: 636–641.

Wunder, T., J. Marr, S. Kremer, O. Sterner, and H. Anke (1997) 1-Methoxypyrene and 1,6-dimethoxypyrene: two novel metabolites in fungal metabolism of polycyclic aromatic hydrocarbons. *Arch. Microbiol.* **167**: 310–316.

Yadav, J.S., and J.C. Loper (2000) Cloning and characterization of the cytochrome P450 oxidoreductase gene from the Zygomycete fungus *Cunninghamella. Biochem. Biophys. Res. Commun.* **268**: 345–353.

Yi, X., M. Mroczko, K.M. Manoj, X. Wang, and L.P. Hager (1999) Replacement of the proximal heme thiolate ligand in chloroperoxidase with a histidine residue. *Proc. Natl. Acad. Sci. USA* **96**: 12412–12417.

Zheng, Z., and J.P. Obbard (2001) Effect of non-ionic surfactants on elimination of polycyclic aromatic hydrocarbons (PAHs) in soil–slurry by *Phanerochaete chrysosporium. J. Chem. Technol. Biotechnol.* **76**: 423–429.

Zheng, Z., and J.P. Obbard (2002a) Oxidation of polycyclic aromatic hydrocarbons (PAH) by the white rot fungus *Phanerochaete chrysosporium. Enzyme Microb. Technol.* **31**: 3–9.

Zheng, Z., and J.P. Obbard (2002b) Removal of surfactant solubilized polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium* in a rotating biological contactor reactor. *J. Biotechnol.* **96**: 241–249.

9

FUNGAL LIGNIN DEGRADATION AND DECOLORIZATION OF PULP AND PAPER MILL EFFLUENTS

9.1   INTRODUCTION

The pulp and paper industry is one of the primary users of wood resources in the world. The purpose of pulping is to extract cellulosics from plant materials and from hard- or softwood trees. At present, three approaches are known to produce pulp from wood: mechanical pulping, chemical pulping, and a combination of both mechanical and chemical pulping. Mechanical pulping provides higher yields and causes less pollution than chemical pulping. The main drawback of mechanical pulping is low-quality pulps, unsuitable for high-strength fiber products and high energy requirements. Chemical pulping involves dissolving lignin from cellulose and hemicellulose fibers. This is known as the *Kraft process*, in which wood chips are cooked in a solution containing sodium hydroxide and sodium sulfide. In this process, less than 50% yields are achieved and the pulp requires extensive bleaching. The *Sulfite process* uses sulfur compounds for pulping, and this pulp requires less bleaching than is required by the Kraft process. Conventional pulp bleaching involves a variety of chlorine species as bleaching agents. During chlorination, wood components such as lignin and some carbohydrates are oxidized and degraded. This is followed by an alkaline extraction phase, which transforms the oxidized products to a soluble form. The final bleaching is performed by oxidizing agents: chlorine dioxide and hydrogen peroxide. All these processes are of environmental concern, due to disposal of the spent chemicals.

Pulp and paper plants are one of the most polluting industries in the world. In India, more than 150 paper and board mills were in operation (Subramanyam, 1990); of these, 36 were large mills with a production capacity of >55 tons/day, and the others were small mills with a production capacity of <30 tons/day (Sastri, 1986). Nearly 175 m^3 of wastewater per ton of manufactured paper is produced by the pulp and paper industry. The bleach plant effluents are of greatest concern, as they release high amounts of chlorinated organics from the chlorine bleaching. One of the main constraints on effluent discharge is the color, which varies from light tan to deep brown to black. One ton of pulp produces about 100 kg of color-generating compounds and 2 to 4 kg of organochlorines to the bleach plant effluents (Nagarathnamma et al., 1999). Effluents from the alkali extraction stage exhibit 80% of the color, 30% of the biochemical oxygen demand (BOD), and 60% of the chemical oxygen demand (COD) of the mill pollution, irrespective of low volume (Prasad and Joyce, 1991).

These effluents cause acute and chronic toxicity. The chlorinated organics in the spent bleaching liquor also show mutagenic qualities (Pacheco and Santos, 1996). The low-molecular-weight chlorolignin fraction contributes to the effluent BOD and acute toxicity, and the high-molecular-weight chlorinated compounds contribute to the effluent color, COD, and chronic toxicity. Color inhibits the natural process of photosynthesis and adversely affects the productivity of the aquatic ecosystem. The end results of the discharge of these effluents include bioaccumulation in fish to the point of impairment of fish quality and diversity in water bodies and various disorders in fish. Regulatory agencies have set up limits to reduce or eliminate these substances completely in discharges. In Scandinavia and North America, chlorine bleaching in pulp mills has been stopped in compliance with regulations on adsorbable organic halides (AOX). The U.S. government has imposed a total ban on AOX discharges, whereas a discharge of up to 1.5 kg of AOX per ton of cellulose pulp is allowed in various parts of Europe (Taseli and Gokcay, 1999). However, chlorine is still used in some pulp plants in Europe and India, due to the low cost and the superior quality of the paper. In India, a few mills use chlorine dioxide for viscosity protection during the first bleaching stage and for brightening during the final bleaching stage. Oxidative extraction and peroxide bleaching are also known in some mills.

9.2   DISTRIBUTION AND STRUCTURE OF LIGNIN

Lignin is a widely distributed and extremely insoluble polymer that provides resistance to microbial attack. It constitutes up to 30% of softwood and 20% of hardwood in the cell walls of Gymnosperms and Angiosperms in association with cellulose and hemicellulose polysaccharides. Lignin is next to cellulose in the global carbon cycle of the biosphere and is the most abundant

material in terms of energy content. Hemicellulose and lignin bind together through cross-links, and the cellulose fibers provide strength.

Lignin is a highly irregular amorphous three-dimensional chemically complex aromatic heteropolymer of *para*-hydroxyphenyl propane units linked through many C—O—C and C—C bonds. The molecular weight of purified lignin ranges from a few thousand to 10^6 Da; in vivo lignin has a molecular weight of 10^5 Da or more. Lignin is randomly synthesized from coniferyl, *para*-coumaryl, and sinapyl alcohol precursors (Figure 9.1). These three precursors differ in the number of methoxy groups on the aromatic ring. The content of these precursors varies in different plant species as well as in different tissues of the same plant. Softwood lignin contains coniferyl and *para*-coumaryl alcohol precursors, and hardwood lignin contains a sinapyl alcohol precursor. Grass and cereal lignin contain covalently bound *para*-hydroxycinnamic acids (i.e., *para*-coumaric and ferulic acids) in addition to the three primary precursors. Lignin cannot be degraded anaerobically and the C—C and ether bonds joining subunits must be cleaved by an oxidative mechanism. Kirk and Brunow (1988) polymerized the lignin precursors in vitro to produce synthetic lignin or dehydrogenative polymerizate (DHP). DHP is free of carbohydrates, soluble in some solvents, and can be labeled with ^{14}C or ^{13}C. DHP is exploited extensively for lignin biodegradation research, despite smaller and varying frequencies of interunit linkages.

An attempt has been made to build a three-dimensional model of lignin structure using a computer program (Jurasek, 1995). The simulated process follows three-dimensional packing of the subunits, and the branched oligomeric structures forms the cross-links. Interchain cross-linking joins the oligomers into one macromolecule and intrachain cross-links result in closed loops. This can result in a macromolecule of molecular weight 3.8×10^5 with an internal density of 1.35g/cm^3. Scanning tunneling microscopy (STM) images of DHP demonstrate a highly ordered process of lignification, even during in vitro conditions, and this contributes to our understanding of the structure of lignin (Radoticacute et al., 1994).
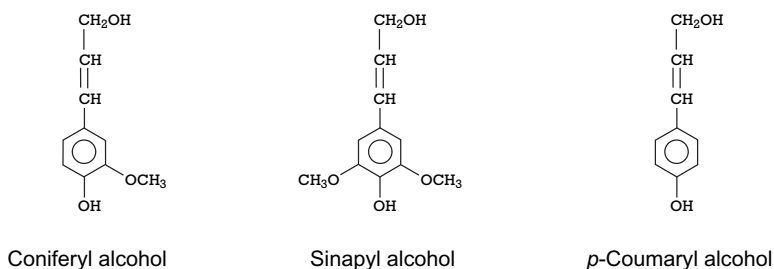
Figure 9.1 Cinnamyl alcohol precursors of lignin and position of carbons.

9.3   LIGNIN-DEGRADING MICROORGANISMS

In nature, lignin degradation is a slow process and takes a number of years. Bacteria, actinomycetes, yeasts, and fungi are known to be involved in lignin degradation. Microbial degradation of lignin has been discussed by many researchers (Garg and Modi, 1999; Blanchette, 2000; Hatakka, 2001).

9.3.1   Bacterial Degradation

Much is not known regarding the degradation of lignin by bacteria. Pure culture studies on bacterial delignification are virtually absent because bacteria cannot grow on cellulose and lignin together. However, certain species of bacteria (e.g., *Aeromonas*, *Arthrobacterium*, *Flavobacterium*, *Pseudomonas*, and *Xanthomonas*) have the ability to degrade lignin (Amer and Drew, 1980; Crawford and Crawford, 1980). Species of *Pseudomonas* are the most efficient degraders of lignin (Vicuna, 1988; Zimmerman, 1990). Nonfilamentous bacteria mineralize less than 10% of synthetic lignin and can metabolize the low-molecular-weight portion of lignin and the degradation products of lignin (Ruttimann et al., 1991; Vicuna et al., 1993). Lignin degradation by thermophilic and anaerobic bacteria has not been demonstrated. Alkali lignin was utilized as the sole source of carbon from sulfate wastewater by species of *Corallina*, *Torula*, *Nocardia*, and *Pseudomonas* (Marchand, 1978). About 98% industrial kraft lignin degrades as the sole carbon source after 5 days of cultivation by *Aeromonas* spp. (Deschamps et al., 1980). Cyanobacteria also plays an important role in the removal of lignin from paper mill effluents (Bharti et al., 1992). Mixed cultures of bacteria, actinomycetes, and fungi in soil and compost can also mineralize lignin (Tuomela et al., 2000).

9.3.2   Fungal Degradation

Fungi are the only microorganisms studied extensively for the degradation of lignin. Lignin degradation by fungi has been discussed by a number of researchers (Evans and Hedger, 2001; Hatakka, 2001). Based on the nature of decay, the wood-rotting fungi are classified in three categories: soft-rot, brown-rot, and white-rot fungi. Soft-rot decay, caused by a number of molds of Ascomycetes and Imperfect Fungi, is known to degrade the major components of wood, including lignin (Blanchette, 1995; Daniel and Nilsson, 1998). Fungi causing soft-rot decay include species of *Allescheria*, *Graphium*, *Monodictys*, *Paecilomyces*, *Papulospora*, and *Thielavia*. Two types of soft rot are recognized: Type I consists of cavities formed within secondary walls, and type II relates to an erosion form of degradation. Better degradation of lignin by these fungi occurs in hardwood than in softwood. Xylariaceous Ascomycetes belonging to the genera *Daldinia*, *Hypoxylon*, and *Xylaria* are now grouped in type II. These fungi occur on hardwood, and 53% of the weight

loss in birch wood occurred within 2 months by *Daldinia concentrica* (Nilsson et al., 1989). The soil fungi *Penicillium chrysogenum*, *Fusarium oxysporum*, and *Fusarium solani* mineralized 27.4%, 23.5%, and 22.6% of ^{14}C-labeled lignin, respectively, from milled wheat straw within 4 weeks (Rodriguez et al., 1996a). Another soil fungus, *Fusarium proliferatum*, mineralized 3.5% of ^{14}C-(ring)-labeled DHP and 10% of [^{14}C$_\beta$] DHP in 30 days (Regaldo et al., 1997). *Fusarium proliferatum* secreted aryl alcohol, and laccase in liquid cultures (Regaldo et al., 1999). *Penicillium chrysogenum* secreted laccase and mineralized 7.9% of [^{14}C$_\beta$] DHP in 29 days (Rodriguez et al., 1996b). A nearly 20% weight loss occurred in pine wood in 3 months by *Chrysonilia sitophila*, with carbohydrate and lignin losses of 18% and 25%, respectively (Rodriguez et al., 1997). Analysis of decayed lignin indicates oxidative C$_\alpha$–C$_\beta$ and β-O-aryl bonds cleavage during lignin degradation. A strain of *Thermoascus aurantiacus* degraded extracts of *Eucalyptus grandis* and bleached *Eucalyptus* kraft pulps (Machuca et al., 1998).

Brown-rot fungi include several species of Basidiomycetes and are most common in softwood. These fungi remove cellulose and hemicellulose from the wood, leaving the lignin as a crumbly brown residue. This is due to lignin demethylation, partial oxidation, and depolymerization. The brown color shows the presence of modified lignin in wood. Lignin can be degraded in softwood and hardwood by these fungi. All brown-rot fungi employ a Fenton-type catalytic system, producing hydroxyl radicals that attack wood components, but there are certain differences in wood decay. Based on the differences in the mechanism, brown-rot fungi are classified into two groups: one belonging to *Gloeophyllum trabeum* and the second including *Coniophora puteana* and *Poria* (*Postia*) *placenta*. *Gloeophyllum trabeum* accumulates oxalic acid, which can be used for the hydrolysis of polysaccharides and as a chelator for a Fe(II)–H$_2$O$_2$ system generating hydroxyl radicals (Shimada et al., 1997). In one study, cultivation of *G. trabeum* on pine wood flakes under optimized conditions led to a 30% release of ^{14}CO$_2$ from ^{14}C-methylated lignin in 52 days (Jin et al., 1990). *Poria placenta* demethoxylated spruce lignin, but there was no ring opening (Davis et al., 1994).

The only and most effective lignin degraders and/or mineralizers are the white-rot fungi or closely related litter-decomposing fungi, which include several hundred species of Basidiomycetes and a few Ascomycetes. These fungi are able to decompose hardwood more extensively than softwood and completely mineralize lignin and carbohydrate components of wood to CO$_2$ and water. Lignin and carbohydrates can be removed at the same proportional rate by some species of white-rot fungi. Selective species of other white-rot fungi remove lignin faster than cellulose. Many white-rot fungi colonize cell lumina, thus causing cell wall erosion. As decay progresses, the eroded areas coalesce and void areas filled with mycelia are formed. This process is known as *nonselective* or *simultaneous rot*; *Trametes versicolor* belongs to this category. Some white-rot fungi decompose lignin without loss of cellulose and create white-pocket rot; *Phellinus nigrolimitatus* belongs to this category.

Ganoderma applanatum and *Heterobasidion annosum* produce both types of attack in the same wood (Eriksson et al., 1990). Litter-decaying Basidiomycetes, such as the genera of *Stropharia* and *Agrocybe*, mineralized only half of the ^{14}C-(ring)-labeled DHP than by wood-inhabiting white-rot fungi (Steffen et al., 1999). Seven white-rot fungi (i.e., *T. versicolor*, *Daedalea flavida*, *Dichomitus squalens*, *Phlebia fascicularia*, *P. floridensis*, *P. radiata*, and *Phanerochaete chrysosporium*) exhibited a 12 to 25% loss of lignin in wheat straw after 32 days (Arora et al., 2002). All fungi produced LiP, MnP, and laccase except *Daedalea flavida* and *Dichomitus squalens*, which lack LiP and MnP, respectively.

White-rot fungi occur more commonly on wood species of Angiosperms than on Gymnosperms (Gilbertson, 1980). Syringyl units are degraded, whereas guaiacyl units are more resistant to degradation. Transmission electron microscopy has revealed partial removal of the middle lamella by *Ceriporiopsis subvermispora* and *Pleurotus eryngii* and removal of lignin from secondary cell walls by *Phlebia radiata* (Burlat et al., 1998). In recent years, more taxonomically diverse fungi have been studied for lignin degradation. In general, it has been found that the physiological process of lignin degradation is fungus-specific and is different from *Phanerochaete chrysosporium*. Differences may be related to the taxonomic position and ecology of fungi. *Ganoderma lucidum* produces MnP in poplar wood but not in pine wood media (D'Souza et al., 1999).

9.4   FUNGAL LIGNIN-DEGRADING ENZYMES

In nature, white-rot fungi are the predominant degraders of lignin, as these fungi contain specific genes for the enzymes necessary for depolymerization of lignin. These enzymes include lignin peroxidase (LiP), manganese peroxidase (MnP), laccase, and hydrogen peroxide–generating enzymes. Reactive oxygen species (ROSs) are also produced and are considered agents of wood decay by fungi. These enzymes are produced in different combinations, suggesting more than one strategy for biodegradation of lignin. The oxidation of phenolic compounds to phenoxy radicals takes place via lignin-degrading enzymes and oxidation of nonphenolic compounds via cation radicals. Based on the pattern of enzyme production, Hatakka (1994) classified the white-rot fungi into three major categories: (1) the lignin–manganese peroxidase group (e.g., *Phanerochaete chrysosporium* and *Phlebia radiata*), (2) the manganese peroxidase–laccase group (e.g., *Dichomitus squalens* and *Rigidoporus lignosus*), and (3) the lignin peroxidase–laccase group (e.g., *Phlebia ochraceofulva* and *Junghuhnia separabilima*).

Phanerochaete chrysosporium has been studied extensively as a model of lignin biodegradation research and production of LiP. Many researchers have recently discussed the oxidative mechanisms, molecular genetics, and applications of a variety of ligninolytic enzymes. A hypothetical mechanism of

lignocellulose transformation by enzymes of white-rot fungi has been described by Leonowicz et al. (2001).

9.4.1 Lignin Peroxidases

Lignin peroxidase (LiP; EC 1.11.1.14) was discovered in *Phanerochaete chrysosporium* (Glenn et al., 1983; Tien and Kirk, 1983) and has become the most thoroughly studied peroxidase. LiPs are produced by most white-rot fungi, such as *Phanerochaete flavido-alba* (Ben Hamman et al., 1999), *Trametes trogii* (Vares and Hatakka, 1997), *Bjerkandera* sp. strain BOS55 (ten Have et al., 1998), *Phlebia ochraceofulva* (Vares et al., 1993), and *Phlebia tremellosa* (Vares et al., 1994). Several isozyme forms have been detected in *P. chrysosporium* cultures and a number of other white-rot fungi (e.g., *Trametes versicolor*, *Bjerkandera adusta*, *Phlebia radiata*). The activity and number of LiP isozymes produced by *P. chrysosporium* vary from 2 to 15, depending on the strain, age of the culture, medium composition, and method of cultivation. LiP activity has not been detected in cultures of all three marine fungi (Raghukumar et al., 1996) or in one of the most active and widely studied biopulping fungus, *Ceriporiopsis subvermispora*.

LiPs are glycosylated heme proteins secreted during secondary metabolism in nutrient-limited cultures. LiPs have a molecular weight of about 40 kDa, a low optimum pH of 2.5 to 3.0, acidic pIs, and a high redox potential. The three-dimensional crystal structure of LiP at 2 to 2.5 Å has also been established (Piontek et al., 1993; Poulos et al., 1993). The substrate binding site of LiP has not been determined with certainty. The crystal structure indicates the heme access channel to be the substrate binding site. The catalytic cycle of LiP is similar to that of other peroxidases. LiP catalyzes the H_2O_2-dependent oxidation of a variety of lignin model compounds in three steps (Martinez, 2002):

$$\text{resting peroxidase } [Fe^{3+} \cdot \text{porphyrin}] + H_2O_2$$
$$\rightarrow \text{compound I } [Fe^{4+} = O \cdot \text{porphyrin}^{\bullet+}] + H_2O$$

$$\text{compound I } [Fe^{4+} = O \cdot \text{porphyrin}^{\bullet+}] + S$$
$$\rightarrow \text{compound II } [Fe^{4+} = O \cdot \text{porphyrin}^{\bullet+}] + S^{\bullet}$$

$$\text{compound II } [Fe^{4+} = O \cdot \text{porphyrin}] + S$$
$$\rightarrow \text{resting peroxidase } [Fe^{3+} \cdot \text{porphyrin}] + S^{\bullet} + H_2O$$
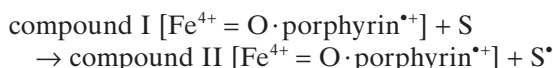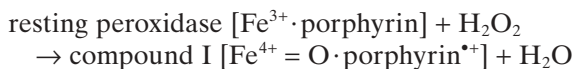
In these reactions, S stands for "substrate" (e.g., veratryl alcohol, β-O-4 lignin dimer, or other lignin substructure). Other fungal enzymes may provide H_2O_2 for LiP activity. White-rot fungi produce H_2O_2-generating oxidases such as glucose oxidases, glyoxal oxidase, and aryl-alcohol oxidase as part of the ligninolytic system. White-rot fungi that lack H_2O_2-generating oxidases employ organic acids such as oxalate and glyoxylate, which produce H_2O_2 indirectly.

The catalytic cycle of LiP is shown in Figure 9.2 (ten Have and Teunissen, 2001).

LiP is relatively nonspecific for reducing substrates, as it reacts with a wide range of lignin model compounds and even unrelated compounds. LiPs can oxidize the cleavage of β-O-4 linkages, C_α–C_β linkages, and other bonds in lignin and its model compounds and are also involved in benzyl alcohol oxidations, side-chain cleavages, ring-opening reactions, demethoxylations, and oxidative dechlorination. Table 9.1 lists some examples of LiP-mediated degradation of lignin. LiP can depolymerize ^{14}C-methylated lignin (Tien and Kirk, 1983) and synthetic lignin, but polymerization occurs simultaneously (Hammel et al., 1993). LiP is unable to oxidize lignin to CO_2 in a cell-free system. The mechanism of LiP action on lignin is still unclear, and it is also not certain that the enzyme can oxidize lignin directly or requires certain radicals, such as VA. LiP has the ability to oxidize ferrocytochrome c (12300 Da), suggesting its action directly on polymeric lignin (Wariishi et al., 1994). Site-directed mutagenesis shows the presence of *Trp* in LiP isozyme H8 that is essential for activity toward VA, as two mutants with different amino acids do not exhibit this activity. Two distinct substrate interaction sites in LiP H8 seem to be present in both mutants (Doyle et al., 1998). A resonance mirror biosensor indicates the specific binding of LiP to synthetic lignin (DHP) (Johjima et al., 1999). It appears that a specific amino acid is responsible for lignin binding. VA and nonphenolic β-O-4 dimeric lignin compound can act as reductants for LiP L3 from *Phlebia radiata* (Lundell et al., 1993a, b). VA plays various roles in the ligninolytic system of *P. chrysosporium*. VA is related to H_2O_2 production in the ligninolytic system of *Pleurotus eryngii* (Guillen and Evans, 1994). Compounds such as 3,4-dimethoxytoluene, 1,4-
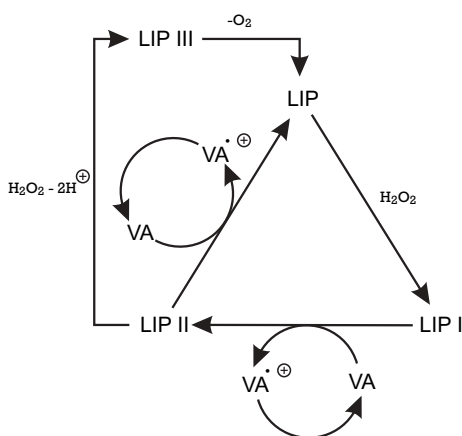
Figure 9.2 Catalytic cycle of lignin peroxidase (LiP). [Reprinted from ten Have and Teunissen (2001), copyright © with permission from the American Chemical Society.]

TABLE 9.1 Peroxidase-Catalyzed Degradation of Lignin

Fungus/Peroxidase	Lignin Model Compound	Medium/Reaction Conditions	Degradation/Mineralization Rate (%)	Metabolic Products	Incubation (hours/weeks)	Reference
Phanerochaete chrysosporium/LiP (crude)	[^{14}C]Methylated spruce lignin	Crude culture liquid, tartrate, H_2O_2, Tween, pH 3.0, 37°C	Depolymerization		1 h	Tien and Kirk, 1983
P. chrysosporium/LiP	^{14}C[DHP] or [α-^{13}C]DHP	Glycolate, VA, 30–40% cosolvent, H_2O_2	Depolymerization	Low-molecular-weight products	16 h	Hammel et al., 1993
P. chrysosporium/LiP, MnP	Hardwood lignin	Aqueous medium, 37°C	5% Degradation		12 h	Thompson et al., 1998
P. chrysosporium/MnP	Four [^{14}C]DHPs	Malonate, 100% O_2, Mn(II), glucose oxidase/glucose, pH 4.5, 37°C	Partially degraded		0.5–7 h	Warriishi et al., 1991
P. chrysosporium/MnP	Chlorolignin	Lactate, Mn(II), H_2O_2	Depolymerization			Lackner et al., 1991
Nematoloma frowardii/MnP	[^{14}C]milled straw lignin	Malonate, pH 4.5, GSH, glucose oxidase/glucose, 37°C	4–10% as $^{14}CO_2$	Low-molecular-weight products	144 h	Hofrichter et al., 1999a
N. frowardii/MnP	[^{14}C-(ring)]DHP	Malonate, fumarate, Mn(II), 100% O_2, 37°C	7% degradation, $^{14}CO_2$ release		192 h	Hofrichter et al., 1999b

TABLE 9.1 *Continued*

Fungus/Peroxidase	Lignin Model Compound	Medium/Reaction Conditions	Degradation/Mineralization Rate (%)	Metabolic Products	Incubation (hours/weeks)	Reference
N. frowardii and *Phlebia radiata*/MnP	[^{14}C-(ring)]DHP	Glucose oxidase/glucose, GSH or not, 100% O$_2$, 37°C	4–6% degradation, ^{14}CO$_2$ release	30–50% Water-soluble products	192 h	Hofrichter et al., 1999c
P. radiata/MnP	[^{14}C-(ring)]DHP	Malonate, Mn(II), pH 4.5, Tween 80, linoleic acid	12(16)% as ^{14}CO$_2$		36(166) h	Kapich et al., 1999a
P. chrysosporium/ Recombinant MnP	Nonphenolic lignin model dimer	MnP-Mn(II)-linoleic acid	20% as ^{14}CO$_2$	Water-soluble products	48 h	Kapich et al., 1999b
N. frowardii	[^{14}C]DHP	Wheat straw, agar plugs, 24°C	59% as ^{14}CO$_2$	10% Products	12 w	Steffen et al., 2000
Pleurotus sp.			59% as ^{14}CO$_2$	11% Products		
Agrocybe praecox			25% as ^{14}CO$_2$	19% Products		
Stropharia coronilla			23% as ^{14}CO$_2$	16% Products		
S. rugosoannulata			23% as ^{14}CO$_2$	16% Products		
S. coronilla	Nonphenolic lignin model compound	MnP-acetate-Mn(II), pH 4.5	50% degradation	Four products	48 h	Kapich et al., 2005

dimethoxybenzene (1,4-DMB), 3,4,5-trimethoxybenzyl alcohol, and 2-Cl-1,4-DMB have been found to substitute the function of VA as a cofactor of LiP (Koduri and Tien, 1994; Joshi and Gold, 1996; Teunissen and Field, 1998).

LiP is a large molecule that penetrates plant cell walls. It appears that exposed regions of the lumen are attacked by LiP to achieve direct degradation. More research is required, but some studies present evidence of degradation of lignin from inside cell walls. Kirk and Cullen (1998) hypothesized on the ability of cell walls to penetrate low-molecular-weight diffusable compounds by LiP, which can oxidize lignin due to indirect oxidation. Oxidation reactions of lignin model compounds catalyzed by LiP have been described by ten Have and Teunissen (2001). This includes the degradation of model compounds accounting for 7% and 50 to 60% of the linkages in lignin.

LiP genes have been characterized in *P. chrysosporium* (de Boer et al., 1987; Asada et al., 1988). Several LiP genes have also been characterized from other species of fungi, including four *Trametes versicolor* clones: LPG1 (Jonsson and Nyman, 1992), LPGII (Jonsson and Nyman, 1994), VLGI (Black and Reddy, 1991), and *Bjerkandera adusta* clones LPO-1 (Asada et al., 1992) and *Phlebia radiata lpg3* (Saloheimo et al., 1989). LiP-like sequences appear to be present in the genomes of *Fomes lignosus* (Huoponen et al., 1990) and *Phlebia brevispora* and *Ceriporiopsis subvermispora* (Ruttiman et al., 1992). In *P. chrysosporium*, LiPs are encoded by a family of at least 10 closely related genes, designated *lipA* through *lipJ* (Gaskell et al., 1994; Cullen, 1997; Steward and Cullen, 1999). LiP genes show a high degree of sequence conservation. Also, multiple peroxidase genes have been found in species of *Pleurotus* and *Ceriporiopsis* (Lobos et al., 1998; Ruiz-Duenas et al., 1999a; Camarero et al., 2000; Tello et al., 2000). The number of introns in genes encoding LiP are eight to nine for *P. chrysosporium* and five to six for *T. versicolor* (Martinez, 2002). Mature protein lengths of LiPs of *B. adusta*, *P. chrysosporium*, *T. versicolor*, and *P. radiata* reveal 349, 342 to 344, 338 to 346, and 337 amino acids, respectively. LiP isozymes from *P. chrysosporium* and *T. versicolor* are related in group III. Putative regulatory elements in the promoter regions of *P. chrysosporium* include xenobiotic response elements (XREs) and cAMP-mediated response elements in LiP genes (Gold and Alic, 1993).

Despite the detection of *lip*-like gene sequences, Eggert et al. (1996a) reported polymerization of lignin preparations rather than polymerization during LiP testing in vitro. Expression of 10 structurally similar LiP genes, *lipA* through *lipJ*, in three linkage groups has been quantified relevant to biopulping (Janse et al., 1998). In contrast to several studies, this investigation focuses on gene expression in wood rather than on cultural conditions. This also demonstrates that the induction of *lipD* is due to carbon starvation, and that of *lipA*, *lipC*, and *lipJ* is due to nitrogen limitation. Genes such as *lipD* and *lipE* are not involved actively in biopulping. The crystal structure of LiP proteins resembles that of cytochrome c oxidase. The overall patterns of *lip* transcripts are different from those of media. Transcript levels from solid

substrates exhibit no correlation with intron structure, genomic organization, or amino acid sequence familarity (Stewart and Cullen, 1999). Variation of gene expression from strain to strain occurs during LiP regulation in *P. chrysosporium* (Kirk and Cullen, 1998).

Heterologous expression of LiP is more difficult than expression of laccase. LiP from *P. chrysosporium* is expressed for the production of apoenzyme in inclusion bodies in *Escherichia coli* with low yields (Doyle and Smith, 1996; Nie et al., 1998). A homologous expression system of LiP H8 of *P. chrysosporium* has been developed under the control of *gpd* promoter (Sollewijn Gelpke et al., 1999).

9.4.2   Manganese Peroxidases

Manganese peroxidase (MnP; EC 1.11.1.13) is secreted in multiple forms in microenvironments by white-rot fungi and certain soil litter–decomposing fungi. A list of 56 fungi that produce MnP in liquid and/or solid-state fermentation has been compiled by Hofrichter (2002). MnP is secreted by a distinct group of Basidiomycetes, such as the families Coriolaceae, Meruliaceae, Polyporaceae, and the soil litter families Strophariaceae and Tricholomataceae. The molecular weight of MnP ranges between 38 and 62.5 kDa, and the molecular weight of the most purified MnP is 45 kDa. About 11 isozymes of MnP are known to be produced by *Ceriporiopsis subvermispora* (Lobos et al., 1994; Urzua et al., 1995). Five isozymes in *P. chrysosporium* MP-1 have been detected to date (Kirk and Cullen, 1998). Like LiP, nitrogen-deficient conditions favor the production of MnP.

MnP is also glycosylated heme-containing extracellular peroxidase. Its catalytic cycle is similar to those of LiP and horseradish peroxidase (HRP), but it uses absolute Mn(II) as a substrate that is widespread in lignocellulose and soil. MnP has a lower redox potential than LiP and is different from other peroxidases, due to the structure of the binding site. Two calcium ions maintain the structure of the active site (Banci, 1997). A catalytic cycle is initiated by binding H_2O_2 or an organic peroxide to the native ferric enzyme, forming an iron–peroxide complex, as depicted in Figure 9.3 (Hofrichter, 2002). Peroxide oxygen–oxygen bond cleaves producing MnP compound I (Fe^{4+}-oxo-porphyrin-radical complex). Subsequent reduction takes place through MnP compound II (Fe^{4+}-oxo-porphyrin complex), and Mn(II) oxidizes to Mn(III), and this continues with the generation of native enzyme and release of the water molecule. MnP compound I is similar to LiP and HRP and can be reduced by electron donors such as ferrocyanide, phenolics, and Mn(II), whereas MnP compound II is very slowly reduced by other substrates and requires Mn(II) for completion of the catalytic cycle (Wariishi et al., 1988). A high concentration of H_2O_2 causes reversible inactivation of MnP by forming compound III, an inactive state. Hybrid forms of MnP and LiP are also known which can oxidize phenolic and nonphenolic compounds. These enzymes are recognized in *Bjerkandera* sp. strain BOS55 (Mester and
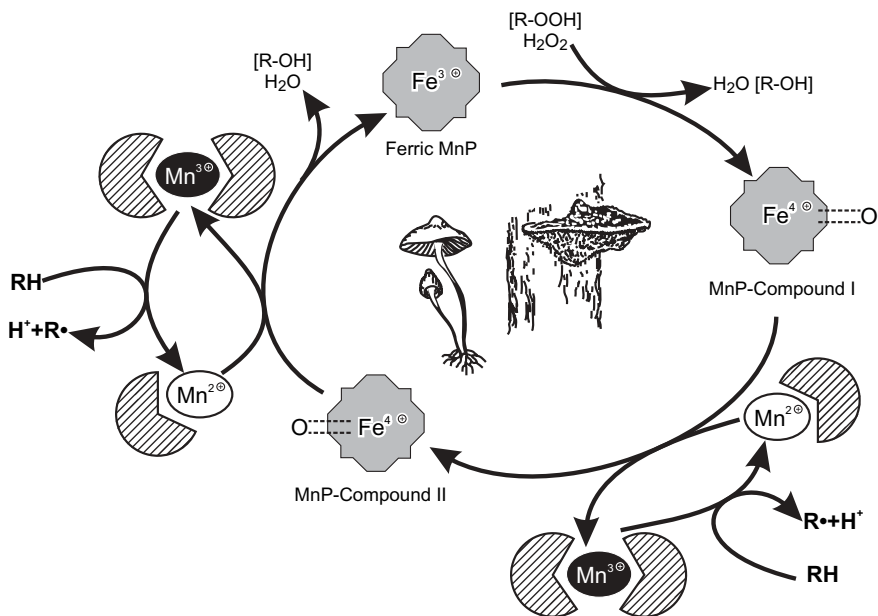
Figure 9.3 The catalytic cycle of manganese peroxidase (MnP). [Reprinted from Hofrichter (2002), copyright © with permission from Elsevier.]

Field, 1998), *Bjerkandera adusta* (Heinfling et al., 1998a, b), and *Pleurotus eryngii* (Martinez et al., 1996; Heinfling et al., 1998b). The crystal structures and molecular models of MnP of *P. chrysosporium* (Sundaramoorthy et al., 1994), *C. subvermispora* (Canales et al., 1998; Tello et al., 2000), and *P. eryngii* (Heinfling et al., 1998b; Camarero et al., 2000) are known based on gene sequences. Crystal structures of substrate binding site mutants of MnP in *P. chrysosporium* at 2.06 Å resolution reveal the presence of only one major Mn binding site (Sundaramoorthy et al., 1997). A revision in molecular biology and the structure–function relationship of MnP has been advocated (Gold et al., 2000).

One-electron oxidation of various substrates can occur by chelates of Mn(III) with organic acids. Chelates of Mn(III) and organic acids react with each other, forming alkyl radicals which undergo spontaneous reactions with dioxygen, producing other radicals, such as superoxide (Hatakka, 2001). These radicals, generated by autocatalytic reactions, appear to be the source of peroxides and can be employed by MnP in the absence of H_2O_2. An MnP system can oxidize organic sulfur-containing cooxidants (e.g., glutathione, L-cystein), unsaturated fatty acids and their derivatives, linoleic acid, and Tween 80 to reactive thiyl and peroxyl radicals. These radicals can attack lignin structures in the presence of dioxygen.

A reaction system containing MnP from *P. chrysosporium*, sodium malonate, Mn(II), and H_2O_2 catalyzes C_α oxidation, C_α–C_β cleavage, and alkyl aryl cleavages of phenolic syringyl type β-1 lignin structures (Wariishi et al., 1989; Tuor et al., 1992). Phenolic substructures are oxidatively cleaved and comprise only a small portion of the total aromatic structures in lignin. Table 9.1 shows some examples of MnP-mediated degradation of lignin. A hypothetical pathway for MnP-catalyzed formation of CO_2 from aromatic lignin structures is shown in Figure 9.4 (Hofrichter, 2002). Increased depolymerization of lignin preparations occurs using both MnP and laccase from *Rigidoporus lignosus* (Galliano et al., 1991), MnP and laccase from *Agaricus bisporus* (Bonnen et al., 1994), MnP and LiP from *P. chrysosporium* (Thompson et al., 1998), and LiP, MnP, and laccase (Martinez-Ingio and Kurek, 1997). A similar approach to the combination of MnP with other extracellular enzymes of white-rot fungi, such as cellobiose dehydrogenase, can be used in the future.

Nonphenolic substructures are abundant in lignin, although unable to undergo oxidation through direct attack by Mn(III)-chelates. Mediators or cooxidants generate reactive radicals which can oxidize such structures. Glutathione oxidizes VA and nonphenolic β-O-4 lignin model dimers, producing, in aryl ether, cleavage of the dimers. MnP from *Phlebia radiata* cleaves the nonphenolic β-O-4 lignin model dimer and catalyzes the mineralization of synthetic lignin in a cell-free system (Kapich et al., 1999a). An MnP–lipid peroxidation system is strong enough to degrade C_α–C_β and β-aryl ether bonds in nonphenolic lignin model dimers (Kawai et al., 1995; Kapich et al., 1999b). Lipid peroxidation of unsaturated lipid formed lipoxyradical intermediates in the presence of Mn(II) that can oxidize nonphenolic model lignin compounds (Kirk and Cullen, 1998). *C. subvermispora* produced benzylic fragments from β-O-4 lignin structures due to MnP lipid-mediated peroxidation in the absence of LiP activity (Jensen et al., 1996). DNA sequence and protein crystallographic examinations reveal the close similarity of MnPs to LiPs. MnP is regulated by the carbon and nitrogen levels in *P. chrysosporium*. In addition, transcription of MnP genes occurs in the presence of Mn(II), whereas Mn(II) does not regulate *mnp3* genes in *P. chrysosporium* and *C. subvermispora* (Gettemy et al., 1998; Tello et al., 2000). Transcription of different MnP isozymes exhibits variable dependencies in the presence of Mn(II) (Gettemy et al., 1998). In *Pleurotus*, proteins with MnP activity are present in cultures lacking Mn(II), and the addition of Mn(II) inhibits the production of MnP (Martinez et al., 1996). MnP gene sequences and cDNA have been cloned and sequenced, including *Trametes versicolor* (Johansson et al., 1993; Johansson and Nyman, 1996), *Pleurotus ostreatus* (Asada et al., 1995a; Giardina et al., 2000; Irie et al., 2000), *Ceriporiopsis subvermispora* (Lobos et al., 1998; Tello et al., 2000), *Dichomitus squalens* (Li et al., 1999), and *Ganoderma applanatum* (Maeda et al., 2001). One MnP encoding gene was found grouped within a 10-kb region in *T. versicolor* (Johansson and Nyman, 1996). Mature protein length shows
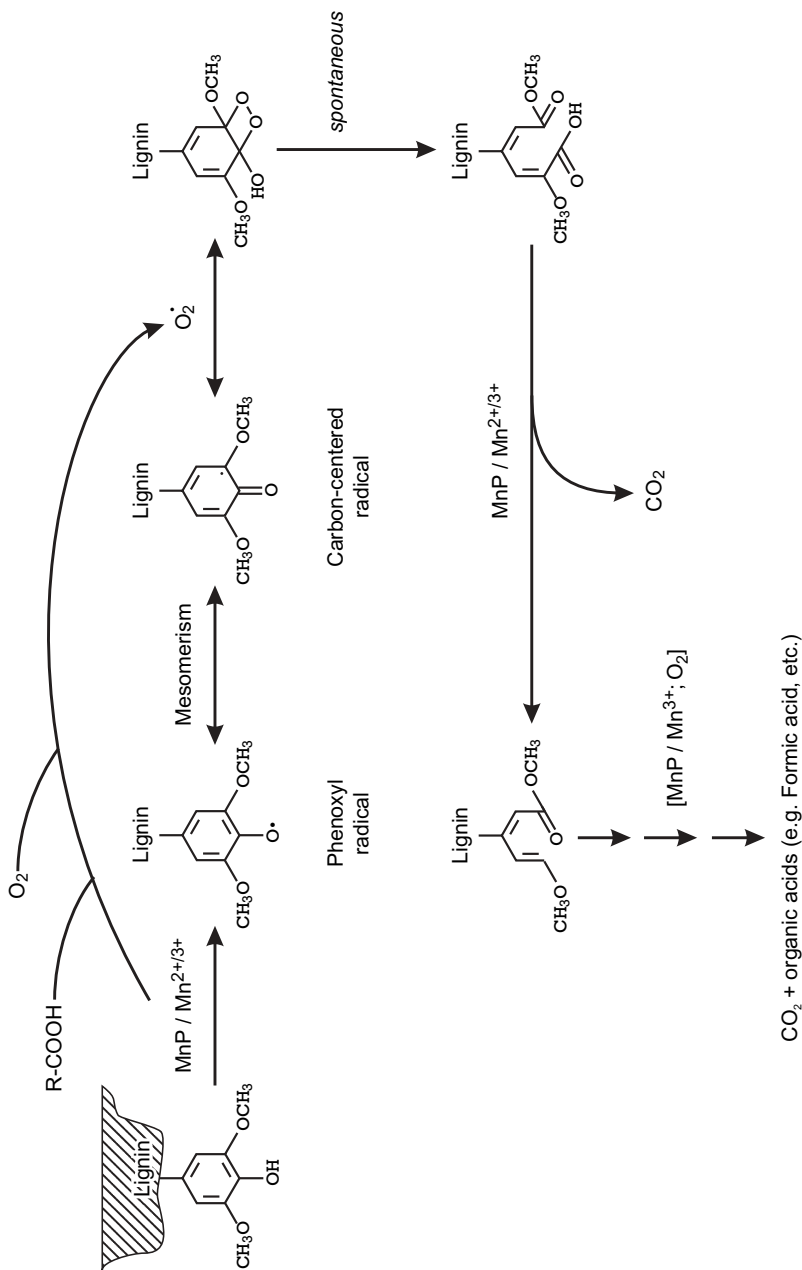
Figure 9.4 Schematic diagram for the formation of carbon dioxide from aromatic lignin structures by MnP. [Reprinted from Hofrichter (2002), copyright © with permission from Elsevier.]

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differences between the MnP of *C. subvermispora* and *D. squalens* (364 to 369 amino acids) and the MnP of *P. chrysosporium* (357 to 358 amino acids). The sequence affinity indicates that eight isozymes from *P. chrysosporium*, *C. subvermispora*, and *D. squalens* are clustered together in group I (Martinez, 2002). Potential transcription control elements are identified in 5′-untranslated regions of MnP genes, and reporter genes can be constructed that confirm these putative regulating elements (Godfrey et al., 1994). Responses from a MnP promotor and applications in determining conditions for optimal MnP gene expression from certain loci have been demonstrated. Putative regulatory elements in the promoter region of a *P. chrysosporium* gene-encoding MnP contain both metal response (MRE) and heat-shock response (HSE) elements (Alic et al., 1997). Heterologous expression of MnP in alternate hosts is more difficult to obtain than LiPs. MnP of *P. chrysosporium* and the *P. eryngii* MnPL2 have been produced successfully as active proteins in various species of *Aspergillus* (Stewart et al., 1996; Ruiz-Duenas et al., 1999b; Conesa et al., 2000).

9.4.3 Laccases

Laccases (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) are widely distributed in many white-rot Basidiomycetes. They are also found in higher plants and in several fungi belonging to Ascomycetes and Deuteromycetes. They are involved in lignin degradation and also serve other functions: in fungal pigmentation, pathogenicity, fructification formation, sporulation, and detoxification.

Laccases are blue glycosylated multi-copper-containing oxidases that are larger than peroxidases and have a molecular weight of 60 to 80 kDa. Laccases contain four coppers per enzyme and are of three different types: type I, type II, and type III. Each type has a distinct role in the oxidation of laccase substrates. Laccases have an optimum pH between 3.0 and 5.7, but some laccases of soil-inhabiting Basidiomycetes have pH optima of 7.0. The optimum temperature is as high as 75ºC for the laccase of the litter-decomposing fungus *Marasmius quercophilus* (Dedeyan et al., 2000). Laccases exhibit significant differences in redox potential (Messerschmidt, 1997). Three types of laccases are classified based on differing redox potential (Eggert et al., 1998). The crystal structure of laccase from *Coprinus cinereus* is resolved at 2.2 Å (Ducros et al., 1998). Laccase from *C. cinereus* expressed *in Aspergillus oryzae* has been crystallized and its cartoonlike 3-D structure has been determined (Ducros et al., 2001).

Laccases can catalyze four single-electron oxidation of phenolic compounds, aromatic amines, and other compounds, coinciding with reduction of molecular O_2 to H_2O. Laccase catalyzes alkyl phenyl and C_α–C_β cleavage of phenolic β-1 and β-O-4 lignin model dimers and demethylation of several lignin model compounds. Laccase is not the key enzyme in lignin degradation, as lignin contains only 10 to 15% phenolic structures in spruce, pine,

and aspen wood (Lai, 1992). Recently, laccase has shown good ability for lignin barrier breakdown in lignocellulose (Leonowicz et al., 2001). *Trametes versicolor* is one of the most efficient laccase-producing fungi. Laccases are also produced in conjunction with LiP, MnP, or both. Laccases from marine fungi effectively mineralized ^{14}C-(ring)-labeled synthetic lignin to $^{14}CO_2$ (Raghukumar et al., 1996). Co-cultivation of white-rot fungi *Cerrena maxima* and *Coriolus hirsutus* revealed the oxidation of a phenolic component of lignin under static and shaken conditions in media with birch sawdust and sulfonate lignin and under solid-state fermentation with birch sawdust (Koroleva et al., 2002). In addition, *C. maxima* produced three laccase isozymes during growth on these media.

Limited use of laccases in lignin degradation is due to size limitations, as it cannot diffuse into pulp fibers. To circumvent these limitations, the use of mediators is postulated. The mediator compounds are also found to enhance the oxidation potential of laccase to nonphenolic moieties of lignin. Common mediators are 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT); less common mediators are polyoxometalate (POM) and *N*-hydroxyacetanilide (NHA). The use of laccase in the presence of mediators to oxidize lignin and delignify kraft pulp is well established (Table 9.2). Many aspects of laccases, including the role of mediators in delignification, have been covered by Argyropoulos (2001).

Neither LiP nor MnP is secreted by *Pycnosporus cinnabarinus*; the only laccase produced is that essential to lignin degradation (Eggert et al., 1996a). *P. cinnabarinus* produces a metabolite as a naturally occurring redox mediator [i.e., 3-hydroxyanthranilate (3-HAA)] for the degradation of nonphenolic lignin and synthetic lignin by laccase (Eggert et al., 1996b). *P. cinnabarinus* appears to be a model system to study alternative strategies for lignin biodegradation. This is the first evidence of a system equivalent to ligninolytic system based on LiP or MnP and the first description of the function of laccase in the complete depolymerization of lignin.

Laccases are produced abundantly by *Trametes versicolor* during pulp bleaching, and at least three forms can be isolated (Paice et al., 1989). Each form binds differently to pulp (Addleman and Archibald, 1993). At least two laccase isoforms (Ruttimann et al., 1992), four or five isozymes (Lobos et al., 1994), and two isozymes, L1 and L2 of laccases (Fukushima and Kirk, 1995) in *Ceriporiopsis subvermispora*, and two laccase isozymes, I and II in *T. versicolor* (Bourbonnais et al., 1995), have been detected. At least seven laccase isozymes have been identified in the white-rot isolate I-62 (Mansur et al., 1997). One of the laccase isozymes from *Pleurotus ostreatus* shows activity inside the cell or in the cell wall (Palmieri et al., 2000). Two laccase isozymes of *Coriolopsis rigida* participate in the production of oxygen free radicals, suggesting their role in the process of lignin degradation (Saparrat et al., 2002).

Several amino acid sequences of laccases have been established. The similarity is about 20 to 60%. Conserved copper-binding sites are found in every

TABLE 9.2 Laccase-Catalyzed Delignification of Lignin

Laccase/Fungus	Mediator	Pulp Type	Delignification (%)	Kappa Reduction (%)	Incubation (hours)	Reference
Trametes versicolor	ABTS	Softwood kraft	40	55	2	Bourbonnais and Paice, 1996
T. versicolor		Sulfite	50			
Laccase I and II	ABTS	Hardwood kraft		12.1 and 12.5		Bourbonnais et al., 1995
Laccase I and II	ABTS	Softwood kraft		22.4 and 22.2		
Polyporus fungus	HBT	Kraft	53		24	Sealey and Ragauskas, 1998
	ABTS		35			
Pycnosporus cinnabarinus	3-HAA	Dimeric lignin model compound	60		48	Eggert et al., 1996b
Coriolus versicolor	HBT	Pine kraft	32	14.8	4	Balakshin et al., 2001a
	POM	*Eucalyptus* kraft	40	8.5	24	Balakshin et al., 2001b
Trametes villosa	VA	Softwood pine kraft		53.6	1	Chakar and Ragauskas, 2001
T. versicolor	NHA	Pine kraft and two-stage O₂-delignified pine kraft	29 / 31	27 / 25	2	Poppius-Levlin et al., 2001
T. versicolor	HBT	Unbleached softwood kraft		21	3	Kandioller and Christov, 2001
Trametes hirsuta	HBT			21		
T. versicolor and *T. hirsuta*	HBT	Post-oxygen softwood kraft		29 and 29		
Pycnosporus sanguineus	HBT			29		
P. sanguineus	HBT	Unbleached hardwood sulfite		51		
T. hirsuta	HBT			51		
T. versicolor and *T. hirsuta*	HBT	Post-oxygen hardwood soda		21 and 23		

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laccase. Based on gene expression, two types of laccases have been distinguished: constitutive and inducible. Copper, 2,5-xylidine, veratric acid, and so on, induce laccase at the level of transcription and translation (Bollag and Leonowicz, 1984; Palmieri et al., 2000). The most effective inducer of laccase is 2,5-xylidine (Leonowicz et al., 2001). More than 17 laccase genes have been cloned and sequenced from different white-rot fungi (Kirk and Cullen, 1998). These include five genes from *Trametes villosa* (Yaver et al., 1996), four from *Rhizoctonia solani* (Wahleithmer et al., 1996), two from *Agaricus bisporus* (Perry et al., 1993), and one each from *Neurospora crassa* (Schilling et al., 1992), *Coriolus hirsutus* (Kojima et al., 1990), *Cryphonectria parasitica* (Choi et al., 1992), *Aspergillus nidulans* (Aramayo and Timberlake, 1990), *Pleurotus ostreatus* (Giardina et al., 1995), and an unidentified basidiomycete (Coll et al., 1993). Laccase gene-specific sequences have been detected in brown-rot fungi (D'Souza et al., 1996). Sequence identity between certain pairs of laccases appears to be low, but conservation is high within regions involved in copper binding. Eleven introns at similar positions have been found in each of three *Rhizoctonia solani* laccase genes (Wahleithmer et al., 1996). Intron position is conserved in the laccase genes of *Trametes villosa* and other Basidiomycetes (Yaver and Golightly, 1996).

Laccase genes are known from at least 15 species of fungi, which includes 22 genes of eight Basidiomycetes (Maijala, 2000). These include *Ceriporiopsis subvermispora* (Karahanian et al., 1998), *Pycnosporus cinnabarinus* (Eggert et al., 1998; Otterbein et al., 2000), several strains of *Trametes versicolor* (Jonsson et al., 1997), *Lentinula edodes* (Zhao and Kwan, 1999), *Coprinus cinereus* (Yaver et al., 1999), and *Phlebia radiata* (Saloheimo and Niku-Paavola, 1991). Nearly 30 laccase genes have been cloned and integrated into various expression vectors and expressed successfully in alternative hosts (Gianfreda et al., 1999). Recombinant laccases have been expressed in industrial hosts such as *Aspergillus oryzae* (Yaver et al., 1999) and the yeast *Pichia pastoris* (Jonsson et al., 1997; Otterbein et al., 2000). However, the first cloning and recombinant laccase of *Myceliophthora thermophila* was expressed in *Aspergillus oryzae* (Berka et al., 1997).

9.4.4 Hydrogen Peroxide–Producing Enzymes

In addition to LiPs, MnPs, and laccases, the white-rot fungi produce a variety of oxidases (e.g., glucose-1-oxidase, glucose-2-oxidase or pyranose-2-oxidase, glyoxal oxidase, methanol oxidase, veratryl alcohol oxidase). These are sources of H_2O_2 and are utilized by peroxidases during the degradation of lignin. Glucose-1-oxidase and glucose-2-oxidase play an important role in the ligninolytic system of *P. chrysosporium* by producing H_2O_2.

Veratryl alcohol (VA) is a secondary metabolite produced by *P. chrysosporium* under nitrogen-limited conditions in parallel with the onset of LiP production. VA also acts as a substrate and stimulates fivefold activity for LiP. VA increases the action of LiP on many substrates, including lignin. Nitrogen

has no effect on VA production in *Bjerkandera* sp. strain BOS55 (Mester et al., 1995). Mester et al. (1995) also showed the inhibition of VA levels by Mn(II) in both *Bjerkandera* sp. strain BOS55 and *P. chrysosporium*. VA is produced via the shikimate pathway, resulting in amino acids such as L-phenylalanine as intermediate. ^{14}C-Isotopic trapping experiments reveal cinnamate, benzoate, and benzaldehyde as the precursors of VA in *P. chrysosporium* and *Bjerkandera* sp. strain BOS55 (Jensen et al., 1994; Mester et al., 1997). Three modes of action of VA are suggested in lignin degradation: (1) a cation radical redox mediator of remote substrates, (2) protection of LiP from inactivation by H_2O_2, and (3) a preferred substrate for the second one-electron reduction of the LiP catalytic cycle. Neither Harvey's mediation (Harvey et al., 1986) nor Valli's protection mechanism (Valli et al., 1990) adequately explains all the veratryl data. However, VA functions as a substrate for compound II, allowing for turnover of the LiP (Zapanta and Tien, 1997). In several white-rot fungi, addition of VA to the culture is found to increase LiP titers. VA can form a cation radical upon oxidation by LiP, and it mediates the oxidation of some substrates. Tryptophan can act in a similar way (Collins et al., 1997). LiP-stabilized VA cation radical can act as a strong redox mediator (Khindaria et al., 1995). Several side oxidation products result in the presence of oxygen, and their formation depends on the hydroperoxyl radical ($\cdot OOH$) by reacting with intermediate radicals (Haemmerli et al., 1987). Recently, the revised mechanism has been discussed on the O_2-dependent formation of side products during oxidation of VA by LiP (ten Have and Franssen, 2001). This mechanism shows that VA reacts first with O_2 and that H_2O attacks thereafter. The inhibitory effect of Mn(II) on VA side product formation is explained in an alternative manner. Mn(II) reducing reactive intermediates to VA has also been proposed.

The extracellular glyoxal oxidase (GLOX; EC 1.2.3.5) is also involved in lignin degradation in *P. chrysosporium*, producing H_2O_2 to catalyze peroxidases (Kersten, 1990). Its action is to transfer electrons from low-molecular-weight aldehydes to O_2, thus forming H_2O_2. The substrates for GLOX include glyoxal and many others. GLOX can be produced on aspen mechanical pulp by *P. chrysosporium* (Datta et al., 1991) or in a chemically defined solid-state fermentation medium by *Pleurotus ostreatus* (Kerem and Hadar, 1996). GLOX genes are cloned from *P. chrysosporium*. GLOX is expressed and secreted in *Aspergillus nidulans* under maltose-inducible expression system at levels 50-fold greater than in *P. chrysosporium* (Kersten et al., 1995).

Cellobiose:quinone oxidoreductase (CBQ; EC 1.1.5.1) is a heme-flavin enzyme that oxidizes cellobiose and some other carbohydrates and reduces a great number of quinones and radicals produced by LiP, MnP, and laccase on lignin model compounds (Ander, 1994). CBQ plays a role in lignin biodegradation in preventing oxidative polymerization of phenols. It also assists in the production of hydroxyl radical by a Fenton cycle (Hyde and Wood, 1997) and creates metal-complexing agents and promotes MnP-mediated Mn(III) production (Roy et al., 1994). In *P. chrysosporium*, CBQ is present under condi-

tions of maximal [^{14}C]DHP degradation (Costa-Ferreira et al., 1994). CBQ originates from the *cdh* gene in *P. chrysosporium* (Raices et al., 2002).

Cellobiose dehydrogenase (CDH; EC 1.1.99.18) is widely produced by many white-rot Basidiomycetes, brown-rot Basidiomycetes in the family of Coniophoraceae, and some soft-rot fungi and molds, and these fungi have been listed by Henriksson et al. (2000). This is an extracellular flavin- and heme-containing enzyme and can reduce O_2 and produce H_2O_2. CDH of white-rot fungi have a molecular weight of 91 to 102 kDa with pIs of 3.8 to 4.2 and an optimum pH of 4.5 to 7.0. CDH and CBQ have many properties in common, including the ability to reduce quinones and oxidize cellobiose and related oligosaccharides. Various quinones and radicals are reduced by CBQ/CDH in white- and brown-rot fungi.

Interaction of CDH/CBQ with quinones and phenoxy radicals produced by LiP, MnP, and laccase during lignin and cellulose degradation has been depicted (Ander and Marzullo, 1997). CBQ/CDH is more closely related to cellulose than lignin degradation. CDH can be employed in the bleaching of pulp, similar to MnP and laccase. CDH degrades cellulose, xylan, and lignin in the presence of H_2O_2 and chelated Fe ions. In addition, different functions of CDH are suggested. These include lignin biodegradation by reducing aromatic radicals generated by ligninolytic enzymes (Temp and Eggert, 1999); support of MnP (Roy et al., 1994); reduction of compound II of ligninolytic peroxidases and completion of the catalytic cycle in the absence of peroxidase substrate (Ander et al., 1993); and degradation and modification of cellulose, hemicellulose, and lignin by generating hydroxyl radicals in a Fenton-type reaction (Kremer and Wood, 1992; Henriksson et al., 1995; Mansfield et al., 1997). In the first step, CDH modifies a nonphenolic benzyl alcohol structure so that MnP can attack (Hilden et al., 2000). Hydroxyl radicals (·OH) generated by cellobiose oxidase eliminate methanol from the aromatic ring, forming a phenolic hydroxy group that is susceptible to MnP attack. Genes encoding CDH are characterized from *P. chrysosporium* (Li et al., 1996), *Trametes versicolor* (Dumonceaux et al., 1998), and *Pycnosporus cinnabarinus* (Moukha et al., 1999). Gene sequences in these fungi are very similar to each other. A cDNA encoding CDH from the wood-rotting fungus *Grifola frondosa* is cloned using RT-PCR, and rapid amplification of cDNA ends (Yoshida et al., 2002). A CDH gene cloned from *T. versicolor* is heterologously expressed in *Pichia pastoris* under the control of *AOX1* methanol inducible promoter (Stapleton et al., 2004). When more information is available, application of these enzymes will be extended to delignification of pulps, pulp bleaching, and degradation of toxic chemicals.

Aryl-alcohol oxidase (AAO; EC 1.1.3.7) is an extracellular FAD-dependent enzyme and a source of H_2O_2 required by peroxidases. This enzyme is found in *P. chrysosporium* (Asada, 1995b) and in liquid cultures of other white-rot fungi [e.g., *Pleurotus sajor-caju* (Bourbonnais and Paice, 1988), *P. eryngii* (Guillen et al., 1990), *P. ostreatus* (Palmieri et al., 1993), and *Bjerkandera adusta* (Muheim et al., 1990)]. AAO from *P. eryngii* is a

glycoprotein with a molecular weight of 72 kDa. AAO from *B. adusta* has two isoforms with molecular weights of 78 kDa and pIs of 4.25 and 4.35. The gene *aao* from *P. eryngii* has been cloned, sequenced, and characterized (Varela et al., 1999). AAO from *Pleurotus pulmonarius* has a molecular weight of 70 kDa with a pI of 3.95, and the cDNA has been cloned and sequenced (Varela et al., 2000).

Aryl-alcohol dehydrogenase (AAD; EC 1.1.1.91) is an intracellular NADP-dependent enzyme found in *P. eryngii* (Guillen and Evans, 1994). This enzyme supplies suitable amounts of aryl alcohol as a source of H_2O_2. AAD appears to be important for the stability of LiP.

9.4.5 Reactive Oxygen Species

It is generally recognized that lignocellulolytic enzymes are too large to penetrate the lignified cell walls of wood, and fungi employ low-molecular-weight agents to initiate decay. These agents are small and diffusible enough to penetrate and may function in the depolymerization of wood cell walls. This hypothesis dates from the middle of the twentieth century, and these agents are known as reactive oxygen species (ROSs). Most of the work is known on hydroxyl radicals ($\cdot$OH), but other ROSs, such as peroxyl radicals (ROO$\cdot$) and hydroperoxyl radicals ($\cdot$OOH), may be involved in fungal attack. Other biological ROS agents include singlet oxygen, hypochlorous acid, and peroxynitrite. ROS appears to be implicated in three major types of wood decay by fungi.

The hydroxyl radical ($\cdot$OH) has received more attention, as this is strongest oxidant in aqueous systems. A pathway is well established for its production via the Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + \cdot OH$). The $\cdot$OH radicals are known to attack on polysaccharides and lignin. Oxidation of lignin with $\cdot$OH results in diverse reactions, some of which can degrade the polymer. These reactions include demethoxylation, hydroxylation, β-O-4 ether cleavage, and C_α oxidation. However, the use of $\cdot$OH to oxidize lignin by any wood decay fungus is still unclear. The peroxyl (ROO$\cdot$) and hydroperoxyl ($\cdot$OOH) are secondary radicals of wood polymers formed via reactions of $\cdot$OH and by some fungal enzymes. MnPs of white-rot fungi produce ROO$\cdot$ due to peroxidation of unsaturated fatty acids (Moen and Hammel, 1994). *C. subvermispora* produces extracellular lipids that can be a source of peroxyl radicals (Enoki et al., 1999). However, more research is required for the role of MnP mechanism in wood decay. These radicals are less reactive and weaker oxidants than $\cdot$OH and may attack lignocellulose. They can abstract benzylic hydrogens at C_α of a lignin side chain and hydrogens from polysaccharides, stabilizing the carbon-center radical due to an adjacent hydroxy or ether oxygen. Peroxyl radicals can also abstract electrons from aromatic ethers those found in lignin similar to those reaction-catalyzed by LiP. These are more selective oxidants than $\cdot$OH, due to nonabstraction of hydrogen from inactivated carbons.

It is difficult to identify ·OH or other ROSs in biological samples. One method used to detect free radicals in cultures is electron spin resonance spectroscopy (Halliwell and Gutteridge, 1999). Another approach is the use of a molecular probe in the cultures that undergoes reactions with an ROS, with the accumulation of products. However, these probes are not specific and many ·OH detectors are oxidized by species other than ·OH, and sometimes to the same products as produced by ·OH. A sensitive and specific fluorescence method has been developed based on the hydroxylation of coumarin-3-carboxylic acid, which produces a fluorescent and stable product, 7-hydroxy-coumarin-3-carboxylic acid (7-OHCCA) (Tornberg and Olsson, 2002). Wood discs colonized by fungi are placed on water agar containing coumarin-3-carboxylic acid, forming 7-OHCCA with the measurement of fluorescence. Eight of 10 species of fungi produce ·OH above the detection limit. A brown-rot fungus produces the highest level: 8 to 25 times the detection limit. Testing of this method in soil reveals 40% recovery of 7-OHCCA with K_2HPO_4 buffer. Wood decay Basidiomycetes also hydroxylate phthalic hydrazide to form a chemiluminescent product (Backa et al., 1992, 1993). Brown- and white-rot fungi show evidence of ·OH production by oxidizing dimethyl sulfoxide to methanesulfinic acid (Hirano et al., 1997; Tanaka et al., 1999). This does not indicate whether the radicals are intracellular or extracellular. Monitoring degradation of recalcitrant polymer, polyethylene glycol (PEG) is used as a general probe for extracellular ROS by growing fungi on wood. PEG can detect the extracellular and nonspecific oxidants produced by fungi grown on natural substrates (Hammel et al., 2002). All of the Basidiomycetes degrade PEG when they can grow on wood. A new assay to estimate ROS production based on the oxidation of a polymeric trap, [14C]phenethyl polyacrylate, is used in cultures of *Gloeophyllum trabeum* and *Postia placenta* (Cohen et al., 2002). Both fungi oxidize aromatic rings on the trap to form significant amounts of 2,5-dimethoxyhydroxyquinone and 2,5-dimethoxy-1,4-benzoquinone and more polar products. 2,5-Dimethoxyhydroquinone is known to be responsible for the Fenton chemistry in vitro. ROS levels are produced in significant amounts in cellulose colonized by both fungi.

During fungal decay, greater ·OH production can occur at a distance from the fungal hyphae and within the lignified secondary cell wall rather than on its surface (Hammel et al., 2002). Low-molecular-weight fungal reductants stable enough to diffuse into wood cell walls are used in situ for the generation of ·OH. The purpose of small fungal metabolites is to reduce Fe(III) to Fe(II) or to reduce O_2 to H_2O_2. Two well-established pathways for ·OH production involve the extracellular reduction of Fe(III) and O_2 by cellobiose dehydrogenase or secreted hydroquinones. Many brown- and white-rot fungi produce oxalic acid, a strong chelator of Fe(III) and Fe(II). If the fungus-colonized wood contains mainly oxalate, redox properties of Fe–oxalate complexes will play a significant role in the Fenton chemistry. A complete Fenton system is delayed until the diffusion of Fe(III) to some distance from the fungal hyphae (Hyde and Wood, 1997; Park et al., 1997). Here the Fenton

reagent is produced within secondary wood cell walls, and this mechanism will protect the fungus from the oxidative damage if ·OH produces outside the hyphae. This model has certain criticisms in that it assumes the formation of H_2O_2 only via Fe(II) autooxidation and requires a pH below 2.5 near the hyphae, even at early stages of decay.

Quinone redox cycling is considered a source of ·OH. In this mechanism, the fungus reduces an extracellular quinone to hydroquinone that reacts with Fe(III) to form Fe(II) and a semiquinone radical. The semiquinone reduces O_2 to produce ·OOH and the original quinone. As ·OOH is a source of H_2O_2, this cycle will produce a complete Fenton system (Kerem et al., 1999). A wide variety of wood decay fungi produce extracellular Fe(III)-binding glycopeptides that may play a role in the Fenton chemistry (Tanaka et al., 1999, 2000). The brown-rot fungus *Gloeophyllum trabeum* employs a quinone redox cycle to produce an extracellular Fenton reagent (Jensen et al., 2002). *Ceriporiopsis subvermispora* produces 1-nonadecene-2,3-dicarboxylic acid, an alkylitaconic acid (ceriporic acid B) that inhibits the formation of ·OH by the Fenton reaction due to direct interaction with Fe ions (Watanabe et al., 2002).

Petriellidium fusoideum produces a protein-free low-molecular-weight substance of about 1.6 kDa containing 0.1% Fe(II) (Gonzales et al., 2002). Fungal laccase in cooperation with this substance generating the hydroxyl radicals participates in the mineralization of synthetic lignin. A hypothetical mechanism is proposed for the generation of ·OH radical in the system in order to describe its participation in the mineralization. Ligninolytic activity of white-rot fungi appears to be related to low-molecular-mass compounds of >5 kDa, in addition to peroxidases and laccases (Moreira et al., 1997). A nonphenolic β-O-4 lignin model dimer has been oxidized to products by three different peroxy radical–generating systems (Kapich et al., 1999b): (1) MnP/Mn(II)/linoleic acid, (2) arachidonic acid in which peroxidation initiates by a small amount of H_2O_2/Fe(II), and (3) thermolysis in air by either 4,4′-azobis(4-cyanovaleric acid) or 2,2′-azobis(2-methylpropionamidine) dihydrochloride. Products exhibited quantitative differences due to the presence of electron-withdrawing substituents on the peroxy radicals derived from azo precursors. Pathways for the oxidation by peroxyl radicals were proposed.

9.4.6 Miscellaneous Enzymes

Oxalate is produced by both white- and brown-rot fungi and acts as an organic acid chelator. It is secreted at the same time as MnP during liquid cultures of *Phanerochaete chrysosporium*. LiP and MnP can decompose oxalate in the presence of VA or Mn(II), respectively. The mechanism involves an electron-transfer reaction between the enzymatically formed VA$^{+•}$ and oxalate, decarboxylating oxalate and reducing VA$^{+•}$ back to VA. Oxalate and malonate are secreted in significant amounts capable of binding Mn(III), making it unavailable to MnP and thus decreasing the rate of Mn(II) oxidation. Strong chela-

tors inhibit the formation of Mn(III)–malonate complex. Stimulation of MnP by chelators is necessary to dissociate Mn(III) from the enzyme because MnP reacts only with Mn(II). Oxalate coexistence with lignin-degrading enzyme systems indicates inhibition in lignin degradation. A small amount of oxalate decreases lignin content significantly in biobleaching experiments. Two new types of extracellular peroxidase, Mn-inhibited peroxidase from *Bjerkandera* sp. strain BOS55 (de Jong et al., 1992) and heme-containing from *Junghuhnia separabilima* (Vares et al., 1992) have been reported, but their role in lignin degradation is still unknown.

9.5 MECHANISMS OF FUNGAL LIGNIN DEGRADATION AND METABOLIC PRODUCTS

Most of the knowledge related to lignin degradation comes from the ligninolytic system and ultrastructure of selective white-rot fungi. Knowledge of the elucidation of mechanisms of degradation of lignin is still far from complete. However, mechanisms of fungal degradation of lignin have been discussed by several researchers and recently by ten Have and Teunissen (2001). The initial steps of lignin degradation are extracellular. The final steps of mineralization are the release of CO_2 that occurs in the fungal hyphae. Lignin is broken into fragments by cellular reactions and diffusing into hyphae. Lignin contains monomers joined by C—C, a bond that is not easily hydrolyzed. Lignin biodegradation is predominantly oxidative in nature; however, reductive reactions also occur. Polymerization of free phenolic groups may occur due to oxidation. Low-molecular-weight fragments are also released. Ligninolytic fungi have the ability to remove low-molecular-weight pieces from the mixture by glycolation or methylation. Lignin demethoxylation occurs using enzyme extracts from *Gloeophyllum trabeum* (Lopretti et al., 1998). Lignin demethylation by two brown-rot fungi, *Postia placenta* and *G. trabeum*, plays a role in polysaccharide decomposition, possibly by assisting in Fenton reactions (Filley et al., 2002). Kirk and Farrell (1987) called the process activated by an enzyme to initiate a thermodynamically favored oxidative fragmentation without control of the reaction pathway *enzymatic combustion*.

Lignin biodegradation leads to the formation of water-soluble intermediates in submerged or solid-state fermentation conditions. Reid (1991) confirmed oligomeric and water-soluble intermediates and products of synthetic lignin (DHP) degradation by *Phlebia tremellosa*. Lignin degradation does not follow an orderly removal of the peripheral subunits, but oxidation of aromatic rings and side chains also takes place. Propyl side chains of β-1 and β-O-4 compounds and aromatic ring openings are cleaved to elucidate the degradation pathways of lignin (Higuchi, 1985, 1993). *P. chrysosporium* and *T. versicolor* cleave alkyl side chains of β-O-4 linked monomethoxyphenylpropane structures to produce dihydroxybenzene products (Vane, 2003).

Ceriporiopsis subvermispora cleaves β-O-4 lignin structures, releasing benzylic fragments by one-electron oxidation mechanism mediated by MnP–lipid peroxidation (Srebotnik et al., 1997). *C. subvermispora* rapidly oxidizes β-5 phenolic dimer (Daina et al., 2002). However, the nonphenolic dimer is not utilized at all even after 21 days under nitrogen-limited and nitrogen-sufficient conditions. A crude MnP is responsible for the degradation of β-5 phenolic compound in vitro. Several oxidation products are detected, but vanillic acid is the predominant degradation product, due to fungal and enzymatic degradation. Crude MnP in the presence of Tween 80 fully oxidizes the methylated β-5 compound. *C. subvermispora* has the ability to decompose lignin without penetration of enzymes into wood cell walls. This fungus produces free 9,12-octadecadienoic, 9-octadecenoic, 11-octadecenoic, hexadecanoic, and octadecanoic acids, predominantly at the incipient stage of cultivation on wood meal cultures (Enoki et al., 1999). During prolonged cultivation of 2 weeks, the amount of intact fatty acids decreases with concomitant production of organic hydroperoxide and thiobarbituric acid reactive substances (TBARS). Immunoelectroscopy revealed the occurrence of ligninolysis at a site far from enzymes. It appears that ligninolysis is catalyzed by free radicals generated at the site of lignin degradation. Nonphenolic lignin is decomposed by a lipid hydroperoxide model in the presence of copper and a fungal metabolite, pyridine (Watanabe et al., 1998). This suggests the production of free radicals for lignin degradation far from enzymes by a combination of metal complexes and lipid hydroperoxides.

White-rot fungi have the ability to degrade lignin in the soil in the presence of cosubstrate, but soil represses the mineralization. These fungi can also degrade lignin linked to humus. Several white-rot fungi degrade synthetic ^{14}C-(ring)-labeled DHP in soil (Tuomela et al., 2002). *Phanerochaete sordida, Trametes versicolor*, and *Phlebia radiata* mineralize 4%, 7%, and 23% of [^{14}C]DHP by inocula in soil, and other fungi mineralize 13 to 17%. Fungi decompose most of [^{14}C]DHP from the dioxane fraction, and some of humic bound [^{14}C]DHP to ^{14}CO$_2$ and water-soluble products. The optimum wheat straw/soil ratio is 1:5 for these fungi. *Trametes hirsuta* mineralizes 30% of [^{14}C]DHP in a straw medium without soil. Most [^{14}C]DHP remains bound to humic substances, especially to humic and fulvic acids, and the binding increases with a decrease in the amount of straw. More information on the mechanisms of lignin degradation was given in the foregoing paragraphs on lignin-degrading enzymes.

9.6 FUNGAL DECOLORIZATION OF PULP AND PAPER MILL EFFLUENTS

The pulp and paper industry is quite old. The color of the wastewaters is due mainly to lignin and lignin derivatives that are discharged from the pulping, bleaching, and chemical recovery operations. Lignin is converted

in the form of thiolignin and alkali lignin during the Kraft process and in the form of lignosulfonate during the Sulfite process. Lignin and its derivatives are difficult to degrade, due to the presence of diphenyl linkages and other bonds. Color is also attributed to double bonds associated with the aromatic ring, quinone methides, and quinone groups. This all implies the need for color removal. Two principal types of chlorine bleach plant wastewaters are produced: the first chlorination (C_1) and the first alkaline extraction stage (E_1) effluents. The major components of C_1-stage effluent are chlorinated phenols; resins and fatty acids; chlorinated hydrocarbons; hypochlorite, chloride, and their degradation products; and those of E_1 are chlorinated and oxidized kraft, and hemicelluloses and their degradation products. The C_1-stage effluents are of low molecular weight, high BOD, low color, toxic, and mutagenic; the E_1-stage effluents are highly colored and contribute 80% of the color, 30% of the BOD, and 60% of the COD of the total mill discharge, although the volume is comparatively low. Effluents from subsequent stages in the bleaching sequence are of relatively minor importance because of their low dissolved organics content and the absence of chlorinated organics. The various effluents—first akali extraction stage E_1, brown stockwasher stage (BSW), combined bleach of all stages, and combined effluent of effluent treatment plant (ETP) from the pulp and paper mill—have been characterized (Mehna et al., 1995). The total pollution load from a bleaching kraft mill is almost equivalent to that of a newsprint factory employing kraft and mechanical pulping. Tissue and speciality paper mills contribute a high pollution load without chemical recovery. Paper board manufacturing discharges a low volume (68 to 90 l/kg of product) of effluent containing high BOD (72 to 82 kg/ton of product) and suspended solids (224 to 290 kg/ton) compared to paper mills (Bajpai and Bajpai, 1994).

Several physical and chemical methods are employed for the removal of color and chloroorganic materials. These include polymeric adsorption, lime coagulation, membrane processes, and rapid land infiltration. Limitations and the expensive nature of these methods lead to a search for alternative methods of treatment. This includes biological decolorization by fungi that can degrade lignin-related phenolic compounds. Fungi, especially white-rot fungi, have been shown to metabolize lignin completely, resulting in rapid decolorization of the effluents. Of 110 pure cultures screened, 10 strains decolorize 75 to 85% of the extraction-stage effluent within 24 to 48 hours (Nagarathnamma et al., 1999). Lignin molecules are polymerized and depolymerized upon treatment of black liquor by *Trametes elegans* (Lara et al., 2003). These effects occur due to ligninolytic activity even in the absence of nutrients. Of eight fungal isolates, the decolorization potency of *Paecilomyces* sp. (F_3) is 67% on the first day, followed by *Phoma* sp. (F_5) and *Paecilomyces variotii* (F_7) for the kraft bleach pulp effluent (Thakur, 2004). Table 9.3 lists the various types of filamentous and white-rot fungi used in the decolorization of pulp and paper mill effluents.

TABLE 9.3 Fungi for Decolorization of Pulp and Paper Mill Effluents

Fungus	Reference
Aspergillus niger	Kannan, 1990
Aspergillus foetidus	Sumathi and Phatak, 1999
Penicillum camemberti	Taseli et al., 2004
Rhizopus oryzae	Nagarathnamma and Bajpai, 1999
Rhizopus pusillus	Driessel and Christov, 2001
Trichoderma sp.	Prasad and Joyce, 1991
Schizophyllum commune	Belsare and Prasad, 1988
Phanerochaete chrysosporium	Bajpai and Bajpai, 1997; Marwaha et al., 1998
Ganoderma lucidum	Perumal et al., 2000
Trametes versicolor	Bajpai et al., 1993; Mehna et al., 1995; Pallerla and Chambers, 1997; Modi et al., 1998
Lentinus (*Lentinula*) *edodes*	Esposito et al., 1991
Marine fungi, *viz.*, *Sordaria fimicola, Halosarpheia ratnagiriensis* and unidentifed basidiomycete fungus no. 312	Raghukumar et al., 1996
Trametes versicolor, Sporotrichum pruinosum, Ceriporiopsis subvermispora CZ-3 and FP105, *Hypodontia setulosa, Phlebia brevispora*, *P. tremellosa, Phellinus pini*, two unidentified strains (EU-6 and EU-10)	Nagarathnamma et al., 1999
Phoma sp. (F$_5$), *Paecilomyces variotii* (F$_7$), isolates of *P. variotii*	Thakur, 2004

9.7 FUNGAL BIOREACTORS FOR DECOLORIZATION OF PULP AND PAPER MILL EFFLUENTS

Fungal bioreactors for the decolorization of pulp and paper mill effluents have been known since the 1980s and are not yet fully developed. Most of the information comes from the application of white-rot fingi in decolorization and degradation of these effluents. However, more knowledge is required, along with the refining of these processes. Fungal bioreactors for the decolorization of these effluents are organized in Table 9.4 and are classified into the categories described below.

9.7.1 Batch and Continuous Bioreactors

Direct use of suspended fungal mycelium is not applicable, due to problems of viscosity, oxygen transfer, and recycling of the fungus. Methods have been

TABLE 9.4 Fungal Bioreactors for Decolorization of Pulp and Paper Mill Effluents

Fungus/Biomass Concentration	Bioreactor Configuration/ Volume	Initial Color Conc. (PCU)	Color/Lignin Removal (%)	COD Removal (%)	Carbon/ Nutrient Addition	Time (hours/ days)	Reference
Trametes versicolor (pellets 3 g/l)	Fermentor, 2.6 L	7000	93	35		48 h	Bajpai et al., 1993
	Packed glass column, 200 ml		93	35		38 h	
T. versicolor (pellets 5 g/l)	Batch 250-ml Erlenmeyer flask	18500 (E_1)	92	69	Sucrose, 5 g/l	7 d	Mehna et al., 1995
	Batch aerobic	7600 (ETP)	82	83		3 d	
Penicillium sp.	Upflow column, 6.7 cm diameter and 55.7 cm height		57		Glucose, 0.05%	10 d	Taseli and Gokcay, 1999
			61		Acetate, 0.2 g/l	7–8 h	
Penicillium camemberti	Upflow column, 6.7 cm diameter and 55.7 cm height		60		Acetate, 0.2 g/l	7–8 h	Taseli et al., 2004
Trichoderma sp.	Disc-rotating, 0.6 mm mesh, 30 cm diameter	2900	57	25		3–18 d	Prasad and Joyce, 1991
Phanerochaete chrysosporium	Immobilized trickling filter		50			3–6 h	Bajpai and Bajpai, 1997
T. versicolor (pellets)	Immobilized on a plastic surface	10000	70		Glucose, 10 g/l	4 d	Martin and Manzanares, 1994
T. versicolor	Ca–alginate immobilized/ Glass cylinder, 600 ml	2357 (EO)	69			1 d	Pallerla and Chambers, 1997
		1842 (caustic and acidic)	61–72			1 d	

TABLE 9.4 Continued

Fungus/Biomass Concentration	Bioreactor Configuration/ Volume	Initial Color Conc. (PCU)	Color/Lignin Removal (%)	COD Removal (%)	Carbon/ Nutrient Addition	Time (hours/ days)	Reference
T. versicolor	Ca–alginate immobilized Polyurethane immobilized Fluidized bed, 600 ml	2472	76–83 69 73			1 d	Pallerla and Chambers, 1995a, b
P. chrysosporium	Jute-rope immobilized Packed bed, 720 cm³		86	54	Glucose and nutrients, 8 g/l Glucose (1% w/v)	3 d	Marwaha et al., 1998
Rhizopus oryzae	Batch 250-ml Erlenmeyer flask Immobilized in polyurethane foam	7266	92–95 95	50 55	Glucose, 1 g/l	24h	Nagarathnamma and Bajpai, 1999
Ceriporiopsis subvermispora	Batch 250-ml Erlenmeyer flask immobilized in polyurethane foam	7000	90 95	45 44	Glucose, 1 g/l	48h	Nagarathnamma et al., 1999
Rhizopus pusillus (42 g)	RBC, 650-ml	6100	71	71		72h	Driessel and Christov, 2001
Coriolus versicolor (39 g)	RBC, 600-ml		53	59			
P. chrysosporium	Batch 100-ml	0.92 g/l (lignin)	62	45	Glucose, 1g/l	10d	Wu et al., 2005
P. ostreatus (biofilm)	Erlenmeyer flask	4.6 g/l (COD) (20% dilution)	70	40	Ammonium tartrate, 0.2 g/l		

developed for the production of pellets and their use in batch and continuous decolorization of such effluents. Pellets of *Coriolus versicolor* can decolorize ultrafiltered kraft liquor with a negligible loss of activity and follow first-order kinetics for the decolorization of bleached kraft effluents (Royer et al., 1991). Fungal pellets are useful in airlift bioreactors to minimize operating costs.

Of three lignin-degrading marine fungi, an unidentified basidiomycete fungus, no. 312, decolorizes 74% of bleach plant effluent at pH 8.2 and 98% at pH 4.5 (Raghukumar et al., 1996). The marine fungi secrete a laccase that is responsible for the effective decolorization of the effluent. Removal of COD coincides with lignin degradation using the biofilms of five white-rot fungi (Wu et al., 2005). Prouty (1990) obtained a maximum decolorization rate of 1300 PCU per day in an aerated reactor using *P. chrysosporium*. The color was reduced to 88% in 48 hours in a laboratory fermentor using *T. versicolor* strain T (Bergbauer et al., 1991). Of three laboratory-scale continuous-flow decolorization bioreactors using *Trichoderma* sp., treatment III was the most attractive, as it decolorized 57% at pH 4.0 after 3 days and maintained over a period of 18 days (Prasad and Joyce, 1991).

9.7.2 Upflow Column Bioreactors

A bench-scale upflow column bioreactor is used for the treatment of paper and pulp effluent (Taseli and Gokcay, 1999; Taseli et al., 2004). The column bioreactor is 6.7 cm in diameter and 55.7 cm in height. It consists of a feed tank, a feed pump, the column itself, an inlet, an outlet, and four sampling ports (Figure 9.5). The column has been operated successfully for 1 to
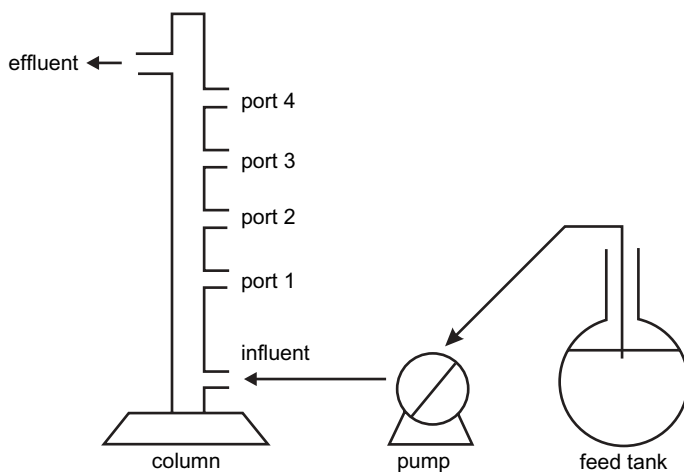
Figure 9.5 Upflow column reactor for the treatment of paper and pulp effluent using *Penicillium camemberti*. [Reprinted from Taseli et al. (2004), copyright © with permission from Elsevier.]

1.5 years with 70% AOX removal in 7 to 8 hours contact with no aeration and a minimum amount of carbon supplement. The highest color removal (60%) and AOX removal (76%) occurred at 0.2 g/l acetate feed concentration.

9.7.3   Immobilized Bioreactors

A considerable amount of work has been done on the use of fungal immobilization to remove effluents. Earlier immobilized systems, rotating biological contactors (RBCs) and trickling filters, employed passive immobilization methods involving adhesion of cells to a solid support. *P. chrysosporium* mycelia on discs of the RBC are used continuously for over 35 days without loss of decolorization activity in the E_1 effluent (Eaton et al., 1982). The fixed film of *P. chrysosporium* FPL/NCSE MyCor is applied for color removal (Campbell et al., 1982). The MyCor process is also successful for the treatment of chlorinated low-molecular-weight phenols of the E_1 effluent (Huynh et al., 1985). A decolorization process for an RBC using whole cells of *P. chrysosporium* resulted in a patent (Chang et al., 1987). A trickling filter system employs polyurethane for immobilization in the treatment of bleach plant effluents in the MyCOPOR process (Messner et al., 1990). Rapid degradation of bleach plant effluent occurs during the first 3 to 6 hours in a trickling filter system, in which immobilized *P. chrysosporium* removes 50% of color and AOX (Bajpai and Bajpai, 1997). This results in a reduction of 80% toxicity against *Daphnia magna*.

Active immobilization techniques include cell entrapment, which can withstand high mechanical resistance and stability against pH and media variations. A cell-free system of *Trametes versicolor* resulted in 56 to 77% continuous decolorization and 41 to 69% AOX reduction in a retention time of 24 hours of pulp and paper wastes (Pallerla and Chambers, 1995a). However, a calcium–alginate polymer bead system with *T. versicolor* resulted in 76 to 83% decolorization and 43 to 59% AOX reduction. A cell-free system of *T. versicolor* decolorized 70% per day, and the decolorization efficiency declined rapidly on the eighth day of the kraft bleach effluents (Pallerla and Chambers, 1995b). However, continuous immobilized bioreactor supports an average of 69% decolorization and 53% AOX reduction within a residence time of 24 hours. About 69% color and 58% AOX are reduced in the caustic-stage effluent in an immobilized bioreactor using calcium–alginate–entrapped *T. versicolor* (Pallerla and Chambers, 1997). Color (61 to 72%) and AOX (54 to 49%) reductions are achieved using a combined effluent (caustic and acidic stages) as the bioreactor feed. The E_1 effluent decolorizes in a continuous bioreactor containing white-rot fungus immobilized on polyurethane foam with removal of 70% color, 64% total phenols, and 7% organic chlorine within 5.8 hours of retention time (Cammarota and SantAnna, 1992). An immobilized *T. versicolor* mycelium darkens progressively due to the accumulation of lignin molecules (Martin

and Manzanares, 1994). Of 80% decolorization, 50% is attributed to adsorption and 30% to fungal degradation.

Jute rope–immobilized *P. chrysosporium* exhibited a better performance than cotton and straw in color removal and COD reduction of an anaerobically digested black liquor (Marwaha et al., 1998). The maximum reduction in color (86%) and COD (54%) was noted up to 3 days and maintained up to the eighth day of the operation and declined thereafter. The reduction in decolorization and COD was due to the deposition of solids on a jute-adsorbed fungal culture. *Rhizopus oryzae* removed 92 to 95% of the color, 50% of the COD, and 72% of the AOX of the alkaline extraction–stage E_1 effluent at 25 to 45°C, pH 3 to 5, within 24 hours (Nagarathnamma and Bajpai, 1999). *Ceriporiopsis subvermispora* CZ-3 removed 90% of the color, 45% of the COD, 62% of the lignin, and 32% of the AOX in 48 hours at 30 to 35°C and pH 4.0 to 4.5 (Nagarathnamma et al., 1999). Immobilized *Rhizopus oryzae* and *Ceriporiopsis subvermispora* mycelia in the polyurethane foam have also been employed repeatedly to treat batches of effluent. During the first batch cycle, the decolorization and COD reduction were 95% and 55% by *Rhizopus oryzae* and 95% and 44% by *Ceriporiopsis subvermispora*, respectively. Decolorization of bleach plant effluent in a RBC by both *Rhizopus pusillus* and *Coriolus versicolor* was directly proportional to the initial color intensities (Driessel and Christov, 2001). The biomass levels in the RBC reactor were 44 g for *C. versicolor* and 68.5 g for *R. pusillus*. *R. pusillus* removed 71% color and COD each and 55% AOX, while *C. versicolor* reduced 53% of the color, 59% of the COD, and 40% of the AOX within 72 hours. Both fungal treatments rendered the effluent nontoxic. Overall, *R. pusillus* was more effective in effluent treatment than *C. versicolor*. MnP and laccase are detected only in *C. versicolor*. Both fungi exhibit differences in the mechanism of color removal, such as adsorption and biodegradation for *C. versicolor* and adsorption for *R. pusillus*.

9.7.4 Miscellaneous Bioreactors

A biomass of *Rhizomucor pusillus*, a thermotolerant fungus, removed 43% and 48% of bleach plant effluent after 2 and 8 hours, respectively (Christov et al., 1999). This fungus can grow and tolerate up to 55°C and exhibits 85% more decolorizing ability at 55°C than at 35°C. This will eliminate the need to cool the effluent for treatment. More than 60% decolorization of E_1-stage effluent occurred using *P. chrysosporium* pellets after 24 hours, which is attributed to rapid adsorption (Jaspers and Penninckx, 1996).

Effluent chromophores of E_1 effluent are destroyed by both ozone and *Coriolus versicolor* treatment, but higher degradation in the form of COD removal is expressed by fungal treatment (Roy-Arcand and Archibald, 1991). Monoaromatic chlorophenolics and toxicity are removed partially by ozone and completely by *C. versicolor*. Both treatments show equal degradation of

all sizes of molecules based on molecular-weight distributions. Partial color removal by ozone treatment followed by fungal treatment provides more effective color removal than fungal pretreatment. Nearly 46 to 53% color is removed by ozone treatment after 24 hours compared to 29% by fungal treatment alone. Biological treatment and post-ozonation are used in mature *Eucalyptus* kraft bleachery effluents (Boyden et al., 1994). A fungal isolate, *Aspergillus*. p2, reduces about 54% of the color in the presence of glucose. Ozonation as a tertiary treatment reduces 60% of the AOX, and the color decrease follows first-order kinetics. Fungal decolorization can be combined with activated sludge of the cotton bleaching effluent (Zhang et al., 1999). First, effluent was decolorized with the mycelial pellets of fungus 7, reducing color by 80%. Second, the decolorized effluent was treated with activated sludge to remove the residual COD. This shows that prior decolorization of the effluent by fungus 7 was easily mineralized by the activated sludge.

In addition to a good fungal selection for effluent treatment, pretreatment is necessary to shorten the retention time for the effluent in a bioreactor. Efficient decolorization was attained by preirradiation of the effluent (15 minutes) at pH 6.5, followed by *Chrysonilia sitophila* culture filtrate treatment (Duran et al., 1991). This decolorization was enhanced by an enzyme cofactor such as hydrogen peroxide. A combination of photochemical and biological methods reduced the COD (Duran, 1992). A combination of photochemical and fungal treatment was also used for the treatment of *Eucalyptus* kraft E_1 effluents (Duran et al., 1994). *Lentinus* (*Lentinula*) *edodes* UEC-2019 removed 73% color of the effluent in 5 days without an additional carbon source. The effluent was preirradiated for 10 minutes in the presence of photocatalyst, ZnO, and followed by *L. edodus* treatment, and this resulted in effective decolorization after 48 hours. Irradiated effluent showed diminished levels of laccase, peroxidase, and β-glucosidase to the half-level. However, LiP and MnP levels were enhanced fourfold. Biomass losses to mineralization were 70% in the biobleaching system and 8% in the preirradiated biobleaching system. Both systems reduced the COD: to 60% and 70%, respectively. The quinone- and carbonyl-type colors were removed more efficiently during fungal decolorization. The combined photo-fungal decolorization treatment appears to be an efficient decontamination process with no color specificity and with a great potential in the industrial effluent treatment.

In a two-step sequential bioreactor, *Paecilomyces* sp. (F_3) reduced 68% of the color of kraft bleached pulp and paper mill effluent within 1 day (Thakur, 2004). However, 82% of the color was reduced on the third day, when fungal-treated effluent was subsequently treated by *Pseudomonas aeruginosa*. Anaerobically treated effluent was applied aerobically in a bioreactor in the presence of *Paecilomyces* sp. (Singh and Thakur, 2005). *Paecilomyces* sp. reduced lignin 86%, color 95%, AOX 67%, phenol 63%, and COD 88% after 3 days.

9.8 FACTORS AFFECTING DECOLORIZATION OF PULP AND PAPER MILL EFFLUENTS

Several factors, including carbon, nitrogen, pH, temperature, dilution of the effluent, inoculum dose, and static versus agitated culture conditions, are known to influence the rate of decolorization of pulp and paper will effluents. Various factors influencing the decolorization of such effluents are noted in Table 9.5.

9.8.1 Carbon Cosubstrate

Glucose is one of the most effective substrates for the decolorization of bleach plant effluents by *Rhizopus oryzae* (Nagarathnamma and Bajpai, 1999), *Trichoderma* sp. (Prasad and Joyce, 1991), and fungus 7 (Zhang et al., 1999). *T. versicolor* removes color efficiently in the presence of sugar refinery or brewery wastes (Archibald et al., 1990). S*chizophyllum commune* reduces nearly 80% of the color in 5 days by the addition of 1% bagasse pith (Ramaswamy, 1987). *T. versicolor* reduced the color about 90% within 3 days with glucose as cosubstrate, accompanied by a 45% reduction in AOX (Bergbauer et al., 1991). Over 50% of total aromatic compounds are degraded. The presence of cosubstrate is necessary for all these activities by the fungus. Successful treatment of E_1 effluent by *Lentinus* (*Lentinula*) *edodes* UEC-2019 resulted in 73% decolorization, reductions of 60% COD, 30% BOD, and 30% phenolics, and only 13% decolorization due to mycelia adsorption without the addition of a carbon or nitrogen source (Esposito et al., 1991). Growth of *Candida utilis* in spent sulfite liquor resulted in a 57% reduction in COD (Streit et al., 1987).

9.8.2 Nitrogen, Phosphorus, Sulfur, and Chloride Concentrations

Increased nitrogen levels inhibit the biodegradation of lignin and derivatives. Higher decolorization rates occur in media containing no NH_4Cl, and the addition of NH_4Cl is deleterious for the decolorization and sustained decolorization under nitrogen-limiting conditions by fungus 7 (Zhang et al., 1999). LiP was produced in media containing high levels of nitrogen. No MnP activity in nitrogen-limited media with added NH_4^+ and no laccase activity occurred in any culture of fungus 7. Nitrogen-limiting conditions or an excess nitrogen supply has no significant effect on the rate of decolorization (Bajpai et al., 1993; Nagarathnamma et al., 1999; Nagarathnamma and Bajpai, 1999). No significant color was removed by the addition of magnesium sulfate by *R. oryzae* (Nagarathnamma and Bajpai, 1999). The maximum color was reduced with 1 g/l of KH_2PO_4 as well as 1.5 g/l of calcium chloride. The maximum color, BOD, and COD reduction using optimum concentrations of sucrose and NH_4Cl were 82%, 63.3% and 75%, respectively, using *Schizophyllum commune* (Belsare and Prasad, 1988). The degradation was completed within

TABLE 9.5 Factors Affecting Fungal Decolorization of Pulp and Paper Mill Effluents

Fungus	Initial Color Conc. (PCU)/ Dilution	Factors/Cosubstrates Conc.	Color Removal (%)	pH	Temperature (°C)	Incubation (hours/days)	Reference
Ceriporiopsis subvermispora	7000	Glucose, 1 g/l	88	4.5	30	96h	Nagarathnamma et al., 1999
		Sucrose, 1 g/l	85				
		Xylose, 1 g/l	87				
		Starch, 1 g/l	86				
		Microcrystalline cellulose, 1 g/l	85				
		Carboxymethyl cellulose, 1 g/l	85				
		Cheese whey, 1 g/l	88				
		Prehydrolysate liquor, 1 g/l	88				
		Ethyl alcohol, 1 g/l	80				
		KH$_2$PO$_4$, 1 g/l	84				
		Magnesium sulfate, 1 g/l	88				
		Calcium chloride, 0.25 g/l	88				
		Medium containing glucose	92				
	4000	Inoculum dose, 0.1 to 4 g/l	25–91				
Rhizopus oryzae	7266	Glucose, 1 g/l	92	4.5	30	24h	Nagarathnamma and Bajpai, 1999
		Microcrystalline cellulose, 1 g/l	90				
		Maltose, 1 g/l	90				
		Sucrose, 1 g/l	89				
		Carboxymethyl cellulose, 1 g/l	88				
		Xylose, 1 g/l	88				
		Starch, 1 g/l	87				
		Ethyl alcohol, 1 g/l	84				
	4000	Medium containing glucose	97				

Organism		Substrate/Medium	%	pH	Temp	Time	Reference
Paecilomyces sp. (F₃)	8333	Minimal salt medium containing Dextrose	82	5.5	30	24h	Thakur, 2004
		Malt, 0.2%	80				
		Pulp, 0.2%	78				
		Corn extract, 0.2%	80				
		Sucrose, 0.2%	78				
		Sodium acetate, 0.2%	72				
Trametes versicolor	7000	Glucose, 5 g/l	84	4.5	30	72h	Bajpai et al., 1993
		Malt extract, 5 g/l	84				
		Ethyl alcohol, 5 g/l	81				
		Starch, 5 g/l	80				
		Pulp, 5 g/l	77				
		Sucrose, 5 g/l	76				
		Inoculum dose, 0.5 to 3 g/l	45–84				
T. versicolor	18500	Sucrose, 5 g/l	82	4.5	30	96h	Mehna et al., 1995
		Glucose, 5 g/l	80				
		Ethyl alcohol, 5 g/l	72				
		Carboxymethyl cellulose, 5 g/l	70				
		Ammonium nitrate, 1.75 g/l	83				
	4125	Medium containing glucose	92				
		Inoculum dose, 0.5 to 5 g/l	45–86				
Trichoderma sp.	2900	Sucrose, 0.05%	43	4.0	30	72h	Prasad and Joyce, 1991
		Pulp (0.45%)	43				144h
		Carboxymethyl cellulose (0.01)	43				
		Sugarcane pith (0.2%)	43				

TABLE 9.5 *Continued*

Fungus	Initial Color Conc. (PCU)/ Dilution	Factors/Cosubstrates Conc.	Color Removal (%)	pH	Temperature (°C)	Incubation (hours/days)	Reference
Fungus 7		Glucose, 5 g/l	72	5.0	30	120h	Zhang et al., 1999
		Cellobiose, 5 g/l	72				
		Starch, 5 g/l	69				
		Maltose, 5 g/l	63				
	20% vol.	NLM with glucose	82				
	30% vol.		75				
		Agitated conditions	70			96h	
T. versicolor	2840	Glucose, 1% and NH$_4$NO$_3$, 0.175 w/v	60	7.8		96h	Modi et al., 1998
Schizophyllum commune	1000	Sucrose, 1 mg/l	60	7.5		120h	Belsare and Prasad, 1988
		Glucose, 1 mg/l	48				
		Sucrose and NH$_4$Cl, 1 mg/l	82				
		Aeration for 5 min every 8h	82–90				
T. versicolor		Glucose	90			72h	Bergbauer et al., 1991
Aspergillus foetidus	2458	Lignin, 0.05%	95	8.0	30	48h	Sumathi and Phatak, 1999
	3874	Lignin, 0.10%	90				
	685 10x	Glucose, 1%	90				
	410 5x	Glucose, 1%	90				
Ganoderma lucidum		IBME medium and inoculum dose, 4 g/l	89	6.5	35	18d	Perumal et al., 2000

2 days and the addition of carbon and nitrogen nutrients does not stimulate further lignin degradation.

9.8.3   Hydrogen Ion Concentration and Temperature

The optimum pH and temperature for decolorization are 5.0 and 27ºC for fungus 7 (Zhang et al., 1999). On the contrary, decolorization occurs at 37 to 39ºC by thermotolerant *P. chrysosporium* (Messner et al., 1990; Feijoo et al., 1995). Practically, decolorization of lignin is nonexistent at 40ºC and weak at temperatures below 32ºC by *C. versicolor* pellets (Royer et al., 1985). *R. pusillus* can grow and retain 85% more decolorizing activities at 55ºC than at 30ºC (Driessel and Christov, 2001). *C. subvermispora* removes nearly 88% of the color at an optimum pH of 4.0 to 4.5 and temperature of 30 to 35ºC (Nagarathnamma et al., 1999). The highest removal of lignin (78%) and COD (78%) occurred at pH 9.0 for *P. chrysosporium* and and 84% and 69%, respectively, for isolate S22 at pH 10.0 (Wu et al., 2005).

9.8.4   Dilution of Effluent

Dilution of the effluent has a strong effect on increased decolorization. The greatest percentage (81%) of color removal occurs at 20% (vol.) cotton bleaching effluent (Zhang et al., 1999). The degradation rate (75%) increases to 30% (vol.) and then remains constant or declines slightly.

9.8.5   Inoculum Dose and Nature

Color removal of combined bleach plant effluents is proportional to an inoculum concentration that results in 60% color reduction at 15% inoculum levels after 6 days of treatment with *C. versicolor* (Eaton et al., 1982). An inoculum dose of 0.5 g/l was optimal for decolorization using *R. oryzae* (Nagarathnamma and Bajpai, 1999). A cool storage time for mycelial pellets of fungus 7 also affects the decolorization activity (Zhang et al., 1999). About 68 to 74% of the color was removed in 4 days by mycelial pellets kept for 1, 3, and 4 months, which is similar to 73% color removal by fresh mycelia in 4 days. However, decolorization activity was lowerd to 36% in 6 days by 6-month-old mycelia. Good storage stability implies the use of mycelial pellets as commercial biocatalysts in color removal processes. The mycelium harvesting time also affects the decolorization (Nagarathnamma et al., 1999). The best decolorization takes place when the mycelium of *C. subvermispora* CZ-3 is harvested after 144 hours of incubation. It also seems that LiP and laccase of *Ganoderma lucidum* play a part in the decolorization of paper mill effluent (Perumal et al., 2000).

9.8.6   Static Versus Agitated Culture Conditions

Poor growth and limited effluent color occur in static cultures (Martin and Manzanares, 1994). Agitation of effluents at 180 rpm results in a significant loss of color with an immobilized *T. versicolor* biomass rather than pellets. Pelleted biomass under submerged cultural conditions contributes to deficient oxygen in the mycelium, thus influencing lower fungal growth and color removal. This implies that the operating conditions can allow a better environment of oxygen diffusion into the medium to prove the proper utility of pellets in decolorization. Decolorization was elevated from 82% to 90%, and BOD and COD were reduced to 70% and 79%, respectively, using *S. commune* after aeration for 5 minutes every 8 hours for 5 days (Belsare and Prasad, 1988).

9.9   EFFECT OF FUNGAL TREATMENT ON CHLOROPHENOLS AND CHLOROALDEHYDES IN EFFLUENTS

Fungal treatment is also known to remove different types of chlorinated phenols and aldehydes in effluents. *C. subvermispora* CZ-3 treatment shows good removal of chlorophenols and chloroaldehydes in E_1-stage effluent (Nagarathnamma et al., 1999). 3-Chlorocatechol, 3,6-dichlorocatechol, 4,5-dichlorocatechol, 3,4,6-trichlorocatechol, tetrachlorocatechol, 3,4,5-trichloroguaiacol, and pentachlorophenol are completely removed after fungal treatment. The percentage removal of 3,6-dichloroguaiacol, 5-chloroguaiacol, 4,5,6-trichloroguaiacol, 6-chloroguaiacol, 2,6-chlorosyringaldehyde, trichlorosyringaldehyde, 4-chlorophenol, and 2-chlorophenol is 96, 94, 73, 67, 64.5, 45.9, 37.8, and 36.5, respectively. The effect of fungal treatment on the elimination of chlorophenols and chloroaldehydes in E_1-stage effluent by *R. oryzae* has also been demonstrated (Nagarathnamma and Bajpai, 1999). The fungal treatment attains complete removal of 2-chlorophenol, 2,6-dichlorophenol, 5-chloroguaiacol, 4,5-dichloroguaiacol, 4,6-dichloroguaiacol, 3,4,5-trichloroguaiacol, 4,5,6-trichloroguaiacol, 4-chlorocatechol, 3,5-dichlorocatechol, 2,6-dichlorosyringaldehyde, and trichlorosyringaldehyde. The percentage removal of 4-chlorophenol, 4,5-dichloroguaiacol, 3,4,6-trichloroguaiacol, 2-chlorosyringaldehyde, and tetrachloroguaiacol is 94, 53, 95, 51, and 58, respectively.

Treatability of chlorinated model compounds such as trichloroacetic acid, 2-chlorophenol, and pentachlorophenol in batch experiments by *Penicillium* sp. suggests better dechlorination of chlorinated aromatics than of aliphatics (Taseli and Gokcay, 1999). Better degradation occurs in ring compounds with numerous chlorine atoms than with fewer chlorine atoms. The molecular-weight distribution before and after fungal treatment demonstrated complete removal of small phenolics (MW <1000), suggesting toxicity reduction in the effluents. A significant reduction in medium-range phenolics (MW: 1000 to

10000) and an increase in the mezo range (MW: 10000 to 30000) were also noted.

The toxicity was assessed by the bioassay test using guppi/zebra fish (Nagarathnamma et al., 1999; Nagarathnamma and Bajpai, 1999). In both cases, the initial LC_{50} (96 hours) of E_1-stage effluent was in the range 50 to 55% by volume. The effluents were nontoxic after respective fungal treatments. This implies the removal of highly toxic compounds such as tri- and tetrachlorophenolic compounds. Nearly 90% color removal and significant toxicity reduction were obtained in CEH bleachery effluents by the Coelomycetous fungus *Stagonospora gigaspora* (Bergbauer et al., 1992).

9.10 DECOLORIZATION OF EFFLUENTS BY FUNGAL ENZYMES

Fungal enzymes, especially peroxidases and laccases, have good potential in decolorization of bleach plant effluents, which contain mostly phenolics and aldehydes. The enzyme activities have been identified related to the effluent (Nagarathnamma et al., 1999). Of three important ligninolytic enzymes, LiP was not detected in the culture fluid or in the mycelium. The maximum laccase and MnP activities were detected on day 6 and 7, respectively, in the culture. The activities of both enzymes in the mycelium were quite low. None of the three ligninolytic enzymes was detected in the culture supernatant of *R. oryzae* (Nagarathnamma and Bajpai, 1999). However, the maximum laccase and MnP activities were detected on day 6 and 7, respectively, in the biomass. MnP plays an important role in the decolorization of effluents by white-rot fungi. LiP and MnP are detected in *Phanerochaete flavido-alba* and appear to be implicated in the decolorization of partially biodepurated paper mill effluents, but LiP activity plays a more important role (Perez et al., 1997). Poor laccase activity by an ascomycete, *Paecilomyces variotii*, significantly decolorizes pulp mill effluent (Calvo et al., 1996).

Fungal enzymes can be immobilized for the decolorization of bleach plant effluents (Table 9.6). Activated silica is employed to immobilize LiP type I, II, III, and lyophilized fungal culture from *Chrysonilia sitophila* (Dezotti et al., 1995). Of these, LiP type III is the most efficient, showing 20% chlorolignin mineralization, 65% COD reduction, and 12% decolorization of *Eucalyptus* kraft effluent. None of these immobilized types showed a loss of activities after being frozen for 4 months or after 5-day contact with the kraft effluent. MnP of *Phanerochaete chrysosporium* appeared to be the major enzyme responsible for 85% decolorization of kraft bleach effluent after 4 days (Michel et al., 1991). Free LiP and lyophilized fungal culture from *Chrysonilia sitophila* decolorized 13% and 6% of kraft effluent, respectively, after 48 hours (Ferrer et al., 1991). Immobilization of LiP type III and lyophilized fungal culture on CNBr–Sepharose 4B decolorized 38% and 29% of kraft effluent, respectively, after 48 hours. It seems that LiPs and lyophilized

TABLE 9.6 Fungal Enzymes for Decolorization of Bleach Plant Effluents

Fungus	Enzyme Concentration	Bioreactor	Initial Color Conc. (PCU)	Color Removal (%)	COD Reduction (%)	pH	Incubation (hours)	Reference
Chrysonilia sitophila	LiP type III 0.003U	Immobilized on activated silica	1900	12	65	5.0	48–120	Dezotti et al., 1995
Lyophilized culture				21	20			
Chrysonilia sitophila	Free LiP, 0.10U LiP III, 0.10U	Immobilized on Sepharose	1900	13 38		5.0	48	Ferrer et al., 1991
Lyophilized culture				6				
Lyophilized culture		Immobilized on Sepharose		29				
Phanerochaete chrysosporium	MnP	High Mn(II) cultures	3000	85		4.5	96	Michel et al., 1991
Coriolus versicolor	Laccase	Immobilized in alginate beads	4000	40			80	Davis and Burns, 1990

fungal culture have potential in treating kraft effluents. The extent of decolorization by soluble and immobilized phenol oxidases and of *C. versicolor* has been compared (Davis and Burns, 1990). Soluble laccase and horseradish peroxidase (HRP) removed color in different effluents, but fast and irreversible enzyme inactivation occurred. Immobilization of these enzymes by entrapped-alginate beads resulted in several-fold decolorization of all effluents. Enzymes are released rapidly in solution, and thus beads could not be employed for continuous purposes. Copolymerization of laccase or HRP with L-tyrosine can lead to the formation of insoluble polymers with enzymatic activity. Entrapment of these copolymers in alginate beads enhanced the decolorization of pulp mill effluent 28 (laccase)- and 132 (HRP)-fold compared with soluble enzymes. Laccase reduced color 40% in 80 minutes.

Enzymatic decolorization of effluents has not met with great success. Many aspects of the mechanisms and functions of fungal enzymes in the decolorization processes of effluents are still unknown. However, decolorization by enzymes has some advantages over fungal treatment. Fungal treatment requires the addition of cosubstrate and adjustment of pH and in certain cases, dilution of the effluent.

9.11 WETLANDS TREATMENT

Wastewater decolorization in constructed wetlands depends on the decomposition activity of microorganisms, especially fungi. It is important to identify and characterize the microbial populations and the dominating colonies in the wetlands. The microbial populations are evaluated in two free-water-surface constructed wetland cells receiving secondary-treated pulp mill wastewater (Hatano et al., 1994). Fungal and actinomycetes populations exhibit small size compared to bacteria in soil or wastewater. About 70% and 50% of fungi show protease and amylase activities, respectively. Fungi exhibit xylanase activity in 40% of isolates. More than 90% of actinomycetes have amylase activity, and 60% and 25% of them have chitinase and xylanase activities, respectively. About 40% of bacteria show protease activity and a few of them have chitinase and xylanase activities. Such microbes can play an important role in reducing the BOD of wastewaters. Unfortunately, the role of constructed wetlands in paper mill effluents by diverse microbial communities has not been given much consideration.

9.12 CONCLUSIONS AND FUTURE PERSPECTIVES

During the past decade, considerable work has been performed on the screening of fungi in lignin degradation and the treatment of pulp and paper mill effluents. The role of fungal enzymes in depolymerization of lignin has been investigated extensively. Fungal enzymes such as LiPs, MnPs, and laccases

have been established for breaking down lignin. Chemical mediators have also been discovered for the laccase-assisted delignification of pulps. The mechanism of lignin solubilization by enzymes is not completely known; however, enzymes at appropriate concentrations can shorten the time required for biopulping and biobleaching. Pathways of lignin biodegradation and its metabolic products have not been determined.

The use of fungi in bioreactors shows success for the decolorization of pulp and paper effluents. By optimizing the requirements, including the coproduction of the relevant enzymes, H_2O_2 and VA, a cost-effective and reliable configured bioreactor can be established, resulting in a higher rate of effluent decolorization. Immobilized bioreactors with sufficient mechanical and chemical stability, extending the activity and lifetime of the biocatalysts, have already shown promise in decolorization and dechlorination of effluents. Clarification of the degradation mechanisms will facilitate the development of novel types of bioreactors. Effluent decolorization by fungal enzymes is still a new area that requires more research.

Despite excellent work on enzymes and genetics of wood degradation by white-rot fungi (Kirk and Cullen, 1998), molecular genetics has not played a significant role in improving the effectiveness of lignin-degrading fungi, leading to shorter reaction times and decolorization and degradation of effluents. Fungal genes have not been isolated specific to meeting these objectives. A 30 million base pair genome of *Phanerochaete chrysosporium* strain RP78 has been sequenced by a whole-genome shotgun method (Martinez et al., 2004). This opens new horizons for a variety of molecular biology studies related to lignin biodegradation. This will include the extended role of various ligninolytic enzymes for the intracellular catabolism of lignin-derived aromatic compounds. Now, it appears that selective effluent decolorizing fungi can be developed that will open a new era in the pulp and paper industry in a few decades. Protein engineering is also being considered to improve the catalytic properties of enzymes that will lead to a new generation of tailor-made ligninolytic enzymes.

REFERENCES

Addleman, K., and F. Archibald (1993) Kraft pulp bleaching and delignification by dikaryons and monokaryons of *Trametes versicolor*. *Appl. Environ. Microbiol.* **59**: 266–273.

Alic, M., L. Akileswaran, and M.H. Gold (1997) Characterization of the gene encoding manganese peroxidase isoenzyme 3 from *Phanerochaete chrysosporium*. *Biochim. Biophys. Acta* **1338**: 1–7.

Amer, G.I., and S.W. Drew (1980) Microbiology of lignin degradation. In: *Annual Report on Fermentation Processes*, G.T. Tsao, ed. Academic Press, New York, Vol. IV, pp. 67–103.

Ander, P. (1994) The cellobiose-oxidizing enzymes CBQ and CbO as related to lignin and cellulose degradation: a review. *FEMS Microbiol. Rev.* **13**: 297–312.

Ander, P., and L. Marzullo (1997) Sugar oxidoreductases and veratryl alcohol oxidase as related to lignin degradation. *J. Biotechnol.* **53**: 115–131.

Ander, P., G. Sena-Martin, and J.C. Duarte (1993) Influence of cellobiose oxidase on peroxidases from *Phanerochaete chrysosporium*. *Biochem. J.* **293**: 431–435.

Aramayo, R., and W.E. Timberlake (1990) Sequence and molecular structure of the *Aspergillus nidulans* yA (laccase I) gene. *Nucleic Acids Res.* **18**: 3415.

Archibald, F.S., M.G. Paice, and L. Jurasek (1990) Decolourization of kraft bleachery effluent chromophores by *Coriolus* (*Trametes*) *versicolor. Enzyme Microb. Technol.* **12**: 846–853.

Argyropoulos, D.S., ed. (2001) *Oxidative Delignification Chemistry: Fundamentals and Catalysis*. ACS Symposium Series 785. American Chemical Society, Washington, DC.

Arora, D.S., M. Chander, and P.K. Gill (2002) Involvement of lignin peroxidase, manganese peroxidase and laccase in degradation and selective ligninolysis of wheat straw. *Int. Biodeterior. Biodegrad.* **50**: 115–120.

Asada, Y., Y. Kimura, and M. Kuwahara (1988) Cloning and sequencing of a ligninase gene from a lignin-degrading basidiomycete, *Phanerochaete chrysosporium. Appl. Microbiol. Biotechnol.* **28**: 469–473.

Asada, Y., Y. Kimura, T. Oka, and M. Kuwahara (1992) Characterization of cDNA and gene encoding a lignin peroxidase from the lignin-degrading basidiomycete *Bjerkandera adusta*. In: *Biotechnology in the Pulp and Paper Industry*, M. Kuwahara and M. Shimada, eds. UNI Publishers, Tokyo, pp. 421–426.

Asada, Y., A. Watanabe, T. Irie, T. Nakayama, and M. Kuwahara (1995a) Structures of genomic and complementary DNAs coding for *Pleurotus ostreatus* manganese (II) peroxidase. *Biochim. Biophys. Acta* **1251**: 205–209.

Asada, Y., A. Watanabe, Y. Ohtsu, and M. Kuwahara (1995b) Purification and characterization of an aryl-alcohol oxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Biosci. Biotechnol. Biochem.* **59**: 1339–1341.

Backa, S., J. Gierer, T. Reitberger, and T. Nilsson (1992) Hydroxyl activity in brown-rot fungi studied by a new chemiluminescence method. *Holzforschung* **46**: 61–67.

Backa, S., J. Gierer, T. Reitberger, and T. Nilsson (1993) Hydroxyl activity associated with the growth of white-rot fungi. *Holzforschung* **47**: 181–187.

Bajpai, P., and P.K. Bajpai (1994) Biological colour removal of pulp and paper mill wastewaters. *J. Biotechnol.* **33**: 211–220.

Bajpai, P., and P.K. Bajpai (1997) Reduction of organochlorine compounds in bleach plant effluents. *Adv. Biochem. Eng. Biotechnol.* **57**: 213–259.

Bajpai, P., A. Mehna, and P.K. Bajpai (1993) Decolorization of kraft bleach plant effluent with the white rot fungus *Trametes versicolor. Process Biochem.* **28**: 377–384.

Balakshin, M., E. Capanema, C.-L. Chen, J. Gratzl, A. Kirkman, and H. Gracz (2001a) Biobleaching of pulp with dioxygen in the laccase-mediator system: reaction mechanisms for degradation of residual lignin. *J. Mol. Catal. B* **13**: 1–16.

Balakshin, M., D.V. Evtuguin, C.P. Neto, and A. Cavaco-Paulo (2001b) Polyoxometalates as mediators in the laccase catalyzed delignification. *J. Mol. Catal. B* **16**: 131–140.

Banci, L. (1997) Structural properties of peroxidases. *J. Biotechnol.* **53**: 253–263.

Belsare, D.K., and D.Y. Prasad (1988) Decolorization of effluent from the bagasse-based pulp mills by white-rot fungus, *Schizophyllum commune. Appl. Microbiol. Biotechnol.* **8**: 301–304.

Ben Hamman, O., T. de la Rubia, and J. Martinez (1999) The effect of manganese on the production of *Phanerochaete flavido-alba* ligninolytic peroxidase in nitrogen limited cultures. *FEMS Microbiol. Lett.* **177**: 137–142.

Bergbauer, M., C. Eggert, and G. Kraepelin (1991) Degradation of chlorinated lignin compounds in a bleach plant effluent by the white rot fungus *Trametes versicolor. Appl. Microbiol. Biotechnol.* **35**: 105–109.

Bergbauer, M., C. Eggert, and G. Kalnowski (1992) Biotreatment of pulp mill bleachery effluents with the coelomycetous fungus *Stagonospora gigaspora. Biotechnol. Lett.* **14**: 317–322.

Berka, R.M., P. Schneider, E.J. Golightly, S.H. Brown, M. Madden, et al. (1997) Characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila* and analysis of the recombinant enzyme expressed in *Aspergillus oryzae. Appl. Environ. Microbiol.* **63**: 3151–3157.

Bharti, S.G., A.S. Salanski, T.C. Taranath, and M.V.R.N. Acharyulu (1992) Role of cyanobacteria in the removal of lignin from paper mill waste waters. *Bull. Environ. Contam. Toxicol.* **49**: 738–742.

Black, A.K., and C.A. Reddy (1991) Cloning and characterization of a lignin peroxidase gene from the white-rot fungus *Trametes versicolor. Biochem. Biophys. Res. Commun.* **179**: 428–435.

Blanchette, R.A. (1995) Degradation of the lignocellulose complex in wood. *Can J. Bot.* **73**(Suppl. I): S999–S1010.

Blanchette, R.A. (2000) A review of microbial deterioration found in archaeological wood from different environments. *Int. Biodeterior. Biodegrad.* **46**: 189–204.

Bollag, J.-M., and A. Leonowicz (1984) Comparative studies of extracellular fungal laccase. *Appl. Environ. Microbiol.* **48**: 849–854.

Bonnen, A.M., L.H. Anton, and A.B. Orth (1994) Lignin-degrading enzymes of the commercial button mushroom, *Agaricus bisporus. Appl. Environ. Microbiol.* **60**: 960–965.

Bourbonnais, R., and M.G. Paice (1988) Veratryl alcohol oxidases from the lignin-degrading basidiomycete *Pleurotus sajor-caju. Biochem. J.* **255**: 445–450.

Bourbonnais, R., and M.G. Paice (1996) Enzymatic delignification of kraft pulp using laccase and a mediator. *TAPPI J.* **79**: 199–204.

Bourbonnais, R., M.G. Paice, I.D. Reid, P. Lanthier, and M. Yaguchi (1995) Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization. *Appl. Environ. Microbiol.* **61**: 1876–1880.

Boyden, B.H., X.Z. Li, T.J. Schulz, O. Hijazin, P. Peiris, and J. Bavor (1994) Treatment of bleachery effluents from kraft mills pulping mature eucalyptus. *Water Sci. Technol.* **29**: 247–258.

Burlat, V., K. Ruel, A.T. Martinez, S. Camarero, A. Hatakka, et al. (1998) The nature of lignin and its distribution in wheat straw affect the patterns of degradation by filamentous fungi. In: *Proceedings of the Seventh International Conference on*

Biotechnology in the Pulp and Paper Industry, Vancouver, BC, Canada, pp. A75–A78.

Calvo, A.M., J.L. Copa, J.B. Fernandez-Larrea, et al. (1996) Improvement in decolorization of paper effluents using ligninolytic fungi and possible enzymes involved. In: *Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry*, E. Srebotnik and K. Messner, eds. Facultas-Universitätsverlag, Vienna, Austria, pp. 253–257.

Camarero, S., F.J. Ruiz-Duenas, S. Sarkar, M.J. Martinez, and A.T. Martinez (2000) The cloning of a new peroxidase found in lignocellulose cultures of *Pleurotus eryngii* and sequence comparison with other fungal peroxidases. *FEMS Microbiol. Lett.* **191**: 37–43.

Cammarota, M.C., and G.L. SantAnna, Jr. (1992) Decolorization of kraft bleach plant E1 stage effluents in a fungal bioreactor. *Environ. Technol.* **13**: 65–72.

Campbell, A.G., Jr., and E.D. Gerrard, T.W. Joyce, H.-M. Chang, and T.K. Kirk (1982) The MyCoR process for color removal from bleach plant effluent: bench scale studies. In: *Proceedings of the Technical Association of the Pulp and Paper Industry Research and Development Conference*, Asheville, NC. TAPPI Press, Atlanta, GA, pp. 209–214.

Canales, M., S. Lobos, and R. Vicuna (1998) Molecular modeling of manganese peroxidase from the lignin-degrading fungus *Ceriporiopsis subvermispora* and structural comparison with other peroxidases. *Electron. J. Biotechnol.* **1**: 96–102.

Chakar, F.S., and A.J. Ragauskas (2001) Formation of quinonoid structures in laccase-mediator reactions. In: *Oxidative Delignification Chemistry: Fundamentals and Catalysis*, D.S. Argyropoulos, ed. ACS Symposium Series 785. American Chemical Society, Washington, DC, pp. 444–456.

Chang, H.-M., T.W. Joyce, and T.K. Kirk (1987) Process of treating effluent from a pulp or paper making operation. U.S. patent 4,655,926.

Choi, G.H., T.G. Larson, and D.L. Nuss (1992) Molecular analysis of the laccase gene from the chestnut blight fungus and selective suppression of its expression in an isogenic hypovirulent strain. *Mol. Plant-Microbe Interact.* **5**: 119–128.

Christov, L.P., B. van Driessel, and C.A. du Plessis (1999) Fungal biomass from *Rhizomucor pusillus* as adsorbent of chromophores from a bleach plant effluent. *Process Biochem.* **35**: 91–95.

Cohen, R., K.A. Jensen, Jr., C.J. Houtman, and K.E. Hammel (2002) Significant levels of extracellular reactive oxygen species produced by brown rot basidiomycetes on cellulose. *FEBS Lett.* **531**: 483–488.

Coll, P.M., C. Tabernero, R. Santamaria, and P. Perez (1993) Characterization and structural analysis of the laccase I gene from the newly isolated ligninolytic basidiomycete PM1 (CECT 2971). *Appl. Environ. Microbiol.* **59**: 4129–4135.

Collins, P.J., J.A. Field, P. Teunissen, and A.D.W. Dobson (1997) Stabilization of lignin peroxidases in white rot fungi by tryptophan. *Appl. Environ. Microbiol.* **63**: 2543–2548.

Conesa, A., C.A.M. van den Hondel, and P.J. Punt (2000) Studies on the production of fungal peroxidases in *Aspergillus niger. Appl. Environ. Microbiol.* **66**: 3016–3023.

Costa-Ferreira, M., P. Ander, and J. Duarte (1994) On the relationship between cellobiose dehydrogenase and cellobiose:quinone oxidoreductase under conditions where [^{14}C]DHP is mineralized by whole cultures of *Phanerochaete chrysosporium*. *Enzyme Microb. Technol.* **16**: 771–776.

Crawford, D.L., and R.L. Crawford (1980) Microbial degradation of lignin. *Enzyme Microb. Technol.* **2**: 11–21.

Cullen, D. (1997) Recent advances in the molecular genetics of ligninolytic fungi. *J. Biotechnol.* **53**: 273–289.

Daina, S., M. Orlandi, G. Bestetti, C. Wiik, and G. Elegir (2002) Degradation of β-5 lignin model dimers by *Ceriporiopsis subvermispora*. *Enzyme Microb. Technol.* **30**: 499–505.

Daniel, G., and T. Nilsson (1998) Developments in the study of soft rot and bacterial decay. In: *Forests Products Biotechnology*, A. Bruce and J.W. Palfreyman, eds. Taylor & Francis, London, pp. 37–62.

Datta, A., A. Bettermann, and T.K. Kirk (1991) Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay. *Appl. Environ. Microbiol.* **57**: 1453–1460.

Davis, S., and R.G. Burns (1990) Decolorization of phenolic effluents by soluble and immobilized phenol oxidases. *Appl. Microbiol. Biotechnol.* **32**: 721–726.

Davis, M.F., H.A. Schroeder, and G.E. Maciel (1994) Solid-state ^{13}C nuclear magnetic resonance studies on wood decay. III. Decay of Colorado blue spruce and paper birch by *Postia placenta*. *Holzforschung* **48**: 301–307.

de Boer, H.A., Y.Z. Zhang, C. Collins, and C. Reddy (1987) Analysis of two nucleotide sequences of two ligninase cDNAs from a white-rot filamentous fungus, *Phanerochaete chrysosporium*. *Gene* **60**: 93–102.

Dedeyan, B., A. Klonowska, S. Taggar, T. Tron, G. Iacazio, et al. (2000) Biochemical and molecular characterization of a laccase from *Marasmius quercophilus*. *Appl. Environ. Microbiol.* **66**: 925–929.

de Jong, E., J.A. Field, and J.A.M. de Bont (1992) Evidence for a new extracellular peroxidase: manganese-inhibited peroxidase from the white-rot fungus *Bjerkandera* sp. BOS55. *FEBS Lett.* **299**: 107–110.

Deschamps, A.M., G. Mahoudeau, and J.M. Lebeault (1980) Fast degradation of kraft lignin by bacteria. *Eur. J. Appl. Microbiol. Biotechnol.* **9**: 45–51.

Dezotti, M., L.H. Innocentini-Mei, and N. Duran (1995) Silica immobilized enzyme catalyzed removal of chlorolignins from eucalyptus kraft effluent. *J. Biotechnol.* **43**: 161–167.

Doyle, W.A., and A.T. Smith (1996) Expression of lignin peroxidase H8 in *Escherichia coli*: folding and activation of the recombinant enzyme with Ca^{2+} and haem. *Biochem. J.* **315**: 15–19.

Doyle, W.A., W. Blodig, N.C. Veitch, K. Piontek, and A.T. Smith (1998) Two substrate interaction sites in lignin peroxidase revealed by site-directed mutagenesis. *Biochemistry* **37**: 15097–15105.

Driessel, B.V., and L. Christov (2001) Decolorization of bleach plant effluents by Mucoralean and white-rot fungi in a rotating biological contactor reactor. *J. Biosci. Bioeng.* **92**: 271–276.

D'Souza, T.M., K. Boominathan, and C.A. Reddy (1996) Isolation of laccase gene-specific sequences from white rot and brown rot fungi by PCR. *Appl. Environ. Microbiol.* **62**: 3739–3744.

D'Souza, T.M., C.S. Merritt, and C.A. Reddy (1999) Lignin-modifying enzymes of the white-rot basidiomycete *Ganoderma lucidum. Appl. Environ. Microbiol.* **65**: 5307–5313.

Ducros, V., A.M. Brzozowski, K.S. Wilson, S.H. Brown, P. Ostergaard, et al. (1998) Crystal structure of the type-2 Cu depleted laccase from *Coprinus cinereus* at 2.2 Å resolution. *Nat. Struct. Biol.* **5**: 310–316.

Ducros, V., A.M. Brzozowski, K.S. Wilson, P. Ostergaard, et al. (2001) The structure of laccase from *Coprinus cinereus* at 1.6 Å resolution: evidence for different type 2 Cu-depleted isoforms. *Acta Crystallogr. D* **57**: 333–336.

Dumonceaux, T.J., K.A Bartholomew, T.C. Charles, S.M. Moukha, and F. S. Archibald (1998) Cloning and sequencing of a gene encoding cellobiose dehydrogenase from *Trametes versicolor. Gene* **210**: 211–219.

Duran, N. (1992) Reduction of chemical oxygen demand in bleach plant effluent by a combination of photochemical and biological methods. In: *Proceedings of the Second Brazil Symposium on the Chemistry of Lignins and Other Wood Components,* N. Duran and E. Esposito, eds. FAPESP, Campinas, Brazil, pp, 323–333.

Duran, N., M. Dezotti, and J. Rodriguez (1991) Biomass photochemistry. XV. Photobleaching and biobleaching of kraft effluent. *J. Photochem. Photobiol. A* **62**: 269–279.

Duran, N., and E. Esposito, L.H. Innocentini-Mei, and V.P. Canhos (1994) A new alternative process for kraft E1 effluent treatment. *Biodegradation* **5**: 13–19.

Eaton, D.C., H.-M. Chang, T.W. Joyce, T.W. Jeffries, and T.K. Kirk (1982) Method obtains fungal reduction of the color of extraction-stage kraft bleach effluents. *TAPPI J.* **65**: 89–92.

Eggert, C., U. Temp, J.F.D. Dean, and K.-El. Eriksson (1996a) A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Lett.* **391**: 144–148.

Eggert, C., U. Temp, and K.-El. Eriksson (1996b) The ligninolytic system of the white-rot fungus *Pycnosporus cinnabarinus*: purification and characterization of the laccase. *Appl. Environ. Microbiol.* **62**: 1151–1158.

Eggert, C., P.R. Lafayette, U. Temp, K.-El. Eriksson, and J.F.D. Dean (1998) Molecular analysis of a laccase gene from the white rot fungus *Pycnosporus cinnabarinus. Appl. Environ. Microbiol.* **64**: 1766–1772.

Enoki, M., T. Watanabe, S. Nakagame, K. Koller, et al. (1999) Extracellular lipid peroxidation of selective white-rot fungus *Ceriporiopsis subvermipora. FEMS Microbiol. Lett.* **180**: 205–211.

Eriksson, K.-El., R.A. Blanchette, and P. Ander (1990) *Microbial and Enzymatic Degradation of Wood and Wood Components*. Springer-Verlag, Berlin.

Esposito, E., V.P. Canhos, and N. Duran (1991) Screening of lignin-degrading fungi for removal of color from kraft mill wastewater with no additional extra carbon source. *Biotechnol. Lett.* **13**: 571–576.

Evans, C.S., and J.N. Hedger (2001) Degradation of plant cell wall polymers. In: *Fungi in Bioremediation*, G.M. Gadd, ed. Cambridge University Press, Cambridge, pp. 1–27.

Feijoo, G., G. Vidal, M.T. Moreira, R. Mendez, and J.M. Lema (1995) Degradation of high molecular weight compounds of kraft pulp mill effluents by a combined treatment with fungi and bacteria. *Biotechnol. Lett.* **17**: 1261–1266.

Ferrer, I., M. Dezotti, and N. Duran (1991) Decolorization of kraft effluent by free and immobilized lignin peroxidases and horseradish peroxidase. *Biotechnol. Lett.* **13**: 577–582.

Filley, T.R., G.D. Cody, B. Goodell, J. Jellison, C. Noser, and A. Ostrofsky (2002) Lignin demethylation and polysaccharide decomposition in spruce sapwood degraded by brown rot fungi. *Org. Geochem.* **33**: 111–124.

Fukushima, Y., and T.K. Kirk (1995) Laccase component of the *Ceriporiopsis subvermispora* lignin-degrading system. *Appl. Environ. Microbiol.* **61**: 872–876.

Galliano, H., G. Gas, J.L. Seris, and A.M. Boudet (1991) Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes: Mn peroxidase and laccase. *Enzyme Microb. Technol.* **13**: 478–482.

Garg, S.K., and D.R. Modi (1999) Decolorization of pulp-paper mill effluents by white-rot fungi. *Crit. Rev. Biotechnol.* **19**: 85–112.

Gaskell, J., P. Stewart, P.J. Kersten, S.F. Covert, J. Reiser, and D. Cullen (1994) Establishment of genetic linkage by allele-specific polymerase chain reaction: application to the lignin peroxidase gene family of *Phanerochaete chrysosporium*. *Bio/Technol.* **12**: 1372–1375.

Gettemy, J.M., B. Ma, M. Alic, and M.H. Gold (1998) Reverse transcription-PCR analysis of the regulation of the manganese peroxidase gene family. *Appl. Environ. Microbiol.* **64**: 569–574.

Gianfreda, L., F. Xu, and J.-M. Bollag (1999) Laccases: a useful group of oxidoreductive enzymes. *Bioremed. J.* **3**: 1–25.

Giardina, P., R. Cannio, L. Martirani, L. Marzullo, G. Palmieri, and G. Sannia (1995) Cloning and sequencing of a laccase gene from the lignin-degrading basidiomycete *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* **61**: 2408–2413.

Giardina, P., G. Palmieri, B. Fontanella, V. Rivieccio, and G. Sannia (2000) Manganese peroxidase isoenzymes produced by *Pleurotus ostreatus* grown on wood sawdust. *Arch. Biochem. Biophys.* **376**: 171–179.

Gilbertson, R.L. (1980) Wood-rotting fungi of North America. *Mycologia* **72**: 1–49.

Glenn, J.K., M.A. Morgan, M.B. Mayfield, M. Kuwahara, and M.H. Gold (1983) An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white-rot basidiomycete *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Commun.* **114**: 1077–1083.

Godfrey, B., L. Akileswaran, and M.H. Gold (1994) A reporter gene construct for studying the regulation of manganese peroxidase gene expression. *Appl. Environ. Microbiol.* **60**: 1353–1358.

Gold, M.H., and M. Alic (1993) Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Microbiol. Rev.* **57**: 605–622.

Gold, M.H., H.L. Youngs, and M.D. Sollewijn Gelpke (2000) Manganese peroxidase. *Met. Ions Biol. Syst.* **37**: 559–586.

Gonzalez, L., J.R. Hernandez, F. Perestelo, A. Carnicero, and M.A. Falcon (2002) Relationship between mineralization of synthetic lignins and the generation of hydroxyl radicals by laccase and a low molecular weight substance produced by *Petriellidium fusoideum*. *Enzyme Microb. Technol.* **30**: 474–481.

Guillen, F., and C.S. Evans (1994) Anisaldehyde and veratraldehyde acting as redox cycling agents for H_2O_2 production by *Pleurotus eryngii*. *Appl. Environ. Microbiol.* **60**: 2811–2817.

Guillen, F., A.T. Martinez, and M.J. Martinez (1990) Production of hydrogen peroxide by aryl-alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii*. *Appl. Microbiol. Biotechnol.* **32**: 465–469.

Haemmerli, S.D., H.E. Schoemaker, H.W.H. Schmidt, and M.S.A. Leisola (1987) Oxidation of veratryl alcohol by the lignin peroxidase of *Phanerochaete chrysosporium*. *FEBS Lett.* **220**: 149–154.

Halliwell, B., and J.M.C. Gutteridge (1999) *Free Radicals in Biology and Medicine*, 3rd ed. Oxford University Press, Oxford.

Hammel, K.E., K.A. Jensen, M.D. Mozuch, L.L. Landucci, M. Tien, and E.A. Pease (1993) Ligninolysis by a purified peroxidase. *J. Biol. Chem.* **268**: 12274–12281.

Hammel, K.E., A.N. Kapich, K.A. Jensen, Jr., and Z.C. Ryan (2002) Reactive oxygen species as agents of wood decay by fungi. *Enzyme Microb. Technol.* **30**: 445–453.

Harvey, P.J., H.E. Schoemaker, and J.M. Palmer (1986) Veratryl alcohol as a mediator and the role of radical cation in lignin biodegradation by *Phanerochaete chrysosporium*. *FEMS Lett.* **195**: 242–246.

Hatakka, A. (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *FEMS Microbiol. Rev.* **13**: 125–135.

Hatakka, A. (2001) Biodegradation of Lignin. In: *Biopolymers*, Vol. 1, *Lignin, Humic Substances and Coal*, M. Hofrichter and A. Steinbuchel, eds. Wiley-VCH, Weinheim, Germany, pp. 129–180.

Hatano, K., D.J. Frederick, and J.A. Moore (1994) Microbial ecology of constructed wetlands used for treating pulp mill wastewater. *Water Sci. Technol.* **29**: 233–239.

Heinfling, A., M.J. Martinez, A.T. Martinez, M. Bergbauer, and U. Szewzyk (1998a) Purification and characterization of peroxidases from the dye-decolorizing fungus *Bjerkandera adusta*. *FEMS Microbiol. Lett.* **165**: 43–50.

Heinfling, A., F.J. Ruiz-Duenas, M.J. Martinez, M. Bergbauer, U. Szewzyk, and A.T. Martinez (1998b) A study on reducing substrates of manganese-oxidizing peroxidases from *Pleurotus eryngii*, and *Bjerkandera adusta*. *FEBS Lett.* **428**: 141–146.

Henriksson, G., P. Ander, B. Pettersson, and G. Pettersson (1995) Cellobiose dehydrogenase (Cellobiose oxidase) as a wood degrading enzyme: studies on cellulose, xylan and synthetic lignin. *Appl. Environ. Microbiol.* **42**: 790–796.

Henriksson, G., G. Johansson, and G. Pettersson (2000) A critical review of cellobiose dehydrogenases. *J. Biotechnol.* **78**: 93–113.

Higuchi, T. (1985) Degradation pathways of lignin model compounds. In: *Biosynthesis and Biodegradation of Wood Components*, T. Higuchi, ed. Academic Press, London, pp. 557–578.

Higuchi, T. (1993) Biodegradation mechanism of lignin by white-rot basidiomycetes. *J. Biotechnol.* **30**: 1–8.

Hilden, L., G. Johansson, L.J. Pettersson, P. Ljungquist, and G. Henriksson (2000) Do the extracellular enzymes cellobiose dehydrogenase and manganese peroxidase form a pathway in lignin biodegradation? *FEBS Lett.* **477**: 79–83.

Hirano, T., H. Tanaka, and A. Enoki (1997) Relationship between production of hydroxy radicals and degradation of wood by the brown-rot fungus, *Tyromyces palustris. Holzforschung* **51**: 389–395.

Hofrichter, M. (2002) Review: lignin conversion by manganese peroxidase (MnP). *Enzyme Microb. Technol.* **30**: 454–466.

Hofrichter, M., K. Scheibner, F. Bublitz, I. Schneegaβ, D. Ziegenhagen, et al. (1999a) Depolymerization of straw lignin by manganese peroxidase from *Nematoloma frowardii* is accompanied by release of carbon dioxide. *Holzforschung* **52**: 161–166.

Hofrichter, M., T. Vares, K. Scheibner, S. Galkin, H. Sipila, and A. Hatakka (1999b) Mineralization and solubilization of synthetic lignin (DHP) by manganese peroxidases from *Nematoloma frowardii* and *Phlebia radiata. J. Biotechnol.* **67**: 217–228.

Hofrichter, M., T. Vares, M. Kalsi, K. Scheibner, and A. Hatakka (1999c) Production of ligninolytic enzymes and organic acids, and mineralization of ^{14}C-labeled lignin during solid-state fermentation of wheat straw with the white-rot fungus *Nematoloma frowardii. Appl. Environ. Microbiol.* **65**: 1864–1870.

Huoponen, K., P. Ollikka, M. Kalin, I. Walther, P. Mantsala, and J. Reiser (1990) Characterization of lignin peroxidase-encoding genes from lignin-degrading basidiomycetes. *Gene* **89**: 145–150.

Huynh, V.-B., H.-M. Chang, and T.W. Joyce (1985) Dechlorination of chloro-organics by a white-rot fungus. *TAPPI J.* **68**: 98–102.

Hyde, S.M., and P. Wood (1997) A mechanism for production of hydroxyl radicals by brown-rot fungus *Coniophora puteana*: Fe(III) reduction by cellobiose dehydrogenase and Fe(II) oxidation at a distance from the hyphae. *Microbiology* **143**: 259–266.

Irie, T., Y. Honda, H.-C. Ha, T. Watanabe, and M. Kuwahara (2000) Isolation of cDNA and genomic fragments encoding the major manganese peroxidase isoenzyme from the white rot basidiomycete *Pleurotus ostreatus. J. Wood Sci.* **46**: 230–233.

Janse, B.J.H., J. Gaskell, M. Akhtar, and D. Cullen (1998) Expression of *Phanerochaete chrysosporium* genes encoding lignin peroxidases, manganese peroxidases, and glyoxal oxidase in wood. *Appl. Environ. Microbiol.* **64**: 3536–3538.

Jaspers, C.J., and M.J. Penninckx (1996) Adsorption effects in the decolorization of a kraft bleach plant effluent by *Phanerochaete chrysosporium. Biotechnol. Lett.* **18**: 1257–1260.

Jensen, K.A., Jr., K.M.C. Evans, T.K. Kirk, and K.E. Hammel (1994) Biosynthetic pathway for veratryl alcohol in the ligninolytic fungus *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **60**: 709–714.

Jensen, K.A., Jr., W. Bao, S. Kawai, E. Srebotnik, and K.E. Hammel (1996) Manganese-dependent cleavage of nonphenolic lignin structures by *Ceriporiopsis*

subvermispora in the absence of lignin peroxidase. *Appl. Environ. Microbiol.* **62**: 3679–3686.

Jensen, K.A., Jr., Z.C. Ryan, A. Vanden Wymelenberg, D. Cullen, and K.E. Hammel (2002) An NADH-quinone oxidoreductase active during biodegradation by the brown-rot basidiomycete *Gloeophyllum trabeum. Appl. Environ. Microbiol.* **68**: 2699–2703.

Jin, I., D.D. Nicholas, and T.K. Kirk (1990) Mineralization of the methoxy carbon of isolated lignin by brown-rot fungi under solid substrate conditions. *Wood Sci. Technol.* **24**: 263–276.

Johansson, T., and P.O. Nyman (1996) A cluster of genes encoding major isozymes of lignin peroxidase and manganese peroxidase fungus from the white-rot fungus *Trametes versicolor. Gene* **170**: 31–38.

Johansson, T., K.G. Welinder, and P.O. Nyman (1993) Isozymes of lignin peroxidase and manganese peroxidase from the white-rot basidiomycete *Trametes versicolor.* 2. Partial sequences, peptide maps, and amino acid and carbohydrate compositions. *Arch. Biochem. Biophys.* **300**: 57–62.

Johjima, T., N. Itoh, M. Kabuto, F. Tokimura, T. Nakagawa, et al. (1999) Direct interaction of lignin and lignin peroxidase from *Phanerochaete chrysosporium. Proc. Natl. Acad. Sci. USA* **86**: 1989–1994.

Jonsson, L., and P.O. Nyman (1992) Characterization of a lignin peroxidase gene from the white-rot fungus *Trametes versicolor. Biochimie* **74**: 177–182.

Jonsson, L., and P.O. Nyman (1994) Tandem lignin peroxidase genes from the fungus *Trametes versicolor. Biochim. Biophys. Acta* **1218**: 408–412.

Jonsson, L., H. Saloheimo, and M. Penttila (1997) Laccase from the white-rot fungus *Trametes versicolor*: cDNA cloning of lcc1 and expression in *Pichia pastoris. Curr. Genet.* **32**: 425–430.

Joshi, D.K., and M.H. Gold (1996) Oxidation of dimethoxylated aromatic compounds by lignin peroxidase from *Phanerochaete chrysosporium. Eur. J. Biochem.* **237**: 45–57.

Jurasek, L. (1995) Towards a three-dimensional model of lignin structure. *J. Pulp Paper Sci.* **21**: J274–J279.

Kandioller, G., and L. Christov (2001) Evaluation of the delignification and bleaching abilities of selected laccases with HBT on different pulps. In: *Oxidative Delignification Chemistry: Fundamentals and Catalysis*, D.S. Argyropoulos, ed. ACS Symposium Series 785. American Chemical Society, Washington, DC, pp. 427–443.

Kannan, K. (1990) Decolourization of pulp and paper mill effluent by growth of *Aspergillus niger. World J. Microbiol. Biotechnol.* **6**: 114–116.

Kapich, A.N., M. Hofrichter, T. Vares, and A. Hatakka (1999a) Coupling of manganese peroxidase-mediated lipid peroxidation with destruction of nonphenolic lignin model compounds and ^{14}C-labeled lignins. *Biochem. Biophys. Res. Commun.* **259**: 212–219.

Kapich, A.N., K.A. Jensen, and K.E. Hammel (1999b) Peroxyl radicals are potential agents of lignin biodegradation. *FEBS Lett.* **461**: 115–119.

Kapich, A.N., K.T. Steffen, M. Hofrichter, and A. Hatakka (2005) Involvement of lipid peroxidation in the degradation of a non-phenolic lignin model compounds

by manganese peroxidase of the litter-decomposing fungus *Stropharia coronilla*. *Biochem. Biophys. Res. Commun.* **330**: 371–377.

Karahanian, E., G. Corsini, S. Lobos, and R. Vicuna (1998) Structure and expression of a laccase gene from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. *Biochem. Biophys. Acta* **1443**: 65–74.

Kawai, S., K.A. Jensen, W. Bao, and K.E. Hammel (1995) New polymeric model substrates for the study of microbial ligninolysis. *Appl. Environ. Microbiol.* **61**: 3407–3414.

Kerem, P.J., and Y. Hadar (1996) Extracellular manganese peroxidase and glyoxal oxidase produced by *Pleurotus ostreatus* in chemically defined SSF. In: *Biotechnology in the Pulp and Paper Industry: Recent Advances in Applied and Fundamental Research*, E. Srebotnik and K. Messner, eds. *Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry*, June 11–15, 1995. Facultas-Universitätsverlag, Vienna, Austria, pp. 369–372.

Kerem, Z., K.A. Jensen, and K.E. Hammel (1999) Biodegradation mechanism of the brown rot basidiomycete *Gloeophyllum trabeum*: evidence for an extracellular hydroquinone-driven fenton reaction. *FEBS Lett.* **446**: 49–54.

Kersten, P.J. (1990) Glyoxal oxidase of *Phanerochaete chrysosporium*: its characterization and activation by lignin peroxidase. *Proc. Natl. Acad. Sci. USA* **87**: 2936–2940.

Kersten, P.J., C. Witek, A. Vanden Wymelenberg, and D. Cullen (1995) *Phanerochaete chrysosporium* glyoxal oxidase is encoded by two allelic variants: structure, genomic organization, heterologous expression of glx1 and glx2. *J. Bacteriol.* **177**: 6106–6110.

Khindaria, A., T.A. Grover, and S.D. Aust (1995) Evidence for the formation of the veratryl alcohol cation radicals by lignin peroxidase. *Biochemistry* **34**: 6020–6025.

Kirk, T.K., and G. Brunow (1988) Synthetic ^{14}C-labeled lignins. *Methods Enzymol.* **161**: 65–73.

Kirk, T.K., and D. Cullen (1998) Enzymology and molecular genetics of wood degradation by white rot fungi. In: *Environmentally Friendly Technologies for the Pulp and Paper Industry*, R.A. Young and M. Akhtar, eds. Wiley, New York, pp. 273–308.

Kirk, T.K., and R.L. Farrell (1987) Enzymatic combustion: the microbial degradation of lignin. *Annu. Rev. Microbiol.* **41**: 465–505.

Koduri, R.S., and M. Tien (1994) Kinetic analysis of lignin peroxidase: explanation for mediation phenomenon by veratryl alcohol. *Biochemistry* **33**: 4225–4230.

Kojima, Y., Y. Tsukuda, Y. Kawai, A. Tsukamoto, et al. (1990) Cloning, sequence analysis, and expression of ligninolytic polyphenoloxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. *J. Biol. Chem.* **265**: 15224–15230.

Koroleva, O.V., V.P. Gavrilova, E.V. Stepanova, I. Lebedeva, et al. (2002) Production of lignin modifying enzymes by co-cultivated white-rot fungi *Cerrena maxima* and *Coriolus hirsutus* and characterization of laccase from *Cerrena maxima*. *Enzyme Microb. Technol.* **30**: 573–580.

Kremer, S., and P. Wood (1992) Production of Fenton's reagent by cellobiose oxidase from cellulolytic cultures of *Phanerochaete chrysosporium*. *Eur. J. Biochem.* **208**: 807–814.

Lackner, R., E. Srebotnik, and K. Messner (1991) Oxidative degradation of high molecular weight chlorolignins by manganese peroxidase of *Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun.* **178**: 1092–1098.

Lai, Y.-Z. (1992) Determination of phenolic hydroxyl groups. In: *Methods in Lignin Chemistry*, S.Y. Lin and C.W. Dence, eds. Springer-Verlag, Berlin, pp. 423–434.

Lara, M.A., A.J. Rodriguez-Malaver, O.J. Rojas, O. Holmquist, et al. (2003) Black liquor lignin biodegradation by *Trametes elegans. Int. Biodeterior. Biodegrad.* **52**: 167–173.

Leonowicz, A., N.-S. Cho, J. Luterek, A. Wilkolazka, et al. (2001) Fungal laccase: properties and activity on lignin. *J. Basic Microbiol.* **43**: 185–227.

Li, B., S.R. Nagalla, and V. Renganathan (1996) Cloning of a cDNA encoding cellobiose dehydrogenase, a hemoflavoenzyme from *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **62**: 1329–1335.

Li, D.M., N. Li, B. Ma, M.B. Mayfield, and M.H. Gold (1999) Characterization of genes encoding two manganese peroxidases from the lignin-degrading fungus *Dichomitis squalens. BBA Prot. Struct. Mol. Enzym.* **1434**: 356–364.

Lobos, S., J. Larram, L. Salas, D. Cullen, and R. Vicuna (1994) Isoenzymes of manganese dependent peroxidase and laccase produced by the lignin degrading basidiomycete *Ceriporiopsis subvermispora. Microbiology* **14**: 2691–2698.

Lobos, S., L. Larrondo, L. Salas, E. Karahanian, and R. Vicuna (1998) Cloning and molecular analysis of a cDNA and the *Cs-mnp1* gene encoding a manganese peroxidase isoenzyme from the lignin-degrading basidiomycete *Ceriporiopsis subvermispora. Gene* **206**: 185–193.

Lopretti, M., D. Cabella, J. Morais, and A. Rodrigues (1998) Demethoxylation of lignin-model compounds with enzyme extracts from *Gloephyllum trabeum. Process Biochem.* **33**: 657–661.

Lundell, T., H. Schoemaker, A. Hatakka, and G. Brunow (1993a) New mechanism of the C_α–C_β cleavage in non-phenolic arylglycerol β-aryl ether lignin substructures catalyzed by lignin peroxidase. *Holzforschung* **47**: 219–224.

Lundell, T., R. Wever, R. Floris, P. Harvey, and A. Hatakka, et al. (1993b) Lignin peroxidase L3 from *Phlebia radiata*: pre-steady-state and steady-state studies with veratryl alcohol and a non-phenolic lignin model compound 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol. *Eur. J. Biochem.* **211**: 391–402.

Machuca, A., H. Aoyama, and N. Duran (1998) Production and characterization of thermostable phenol oxidases of the ascomycete *Thermoascus aurantiacus. Biotechnol. Appl. Biochem.* **27**: 217–223.

Maeda, Y., S. Kajiwara, and K. Ohtaguchi (2001) Manganese peroxidase gene of perennial mushroom *Elfvingia applanata*: cloning and evaluation of its relationship with lignin degradation. *Biotechnol. Lett.* **23**: 103–109.

Maijala, P. (2000) *Heterobasidion annosum* and wood-decay: enzymology of cellulose, hemicellulose, and lignin degradation. Dissertation, Biocentri Viikki Universitatis Helsingiensis, Helsinki, Finland.

Mansfield, S.D., E. de Jong, and J.N. Saddler (1997) Cellobiose dehydrogenase, an active agent in cellulose depolymerization. *Appl. Environ. Microbiol.* **63**: 3804–3809.

Mansur, M., T. Suarez, J.B. Fernandez-Larrea, M.A. Brizuela, and A.E. Gonzalez (1997) Identification of a laccase gene family in the new lignin-degrading basidiomycete CECT 20197. *Appl. Environ. Microbiol.* **63**: 2637–2646.

Marchand, M. (1978) Lignolytic activity of some microorganisms isolated from clarification plant for paper-mill effluents. *Rev. Ecol. Biol. Sol.* **15**: 323–331.

Martin, C., and P. Manzanares (1994) A study of the decolorization of straw soda-pulping effluents by *Trametes versicolor. Bioresour. Technol.* **47**: 209–215.

Martinez, A.T. (2002) Molecular biology and structure-function of lignin-degrading heme peroxidases. *Enzyme Microb. Technol.* **30**: 425–444.

Martinez, M.J., G.F. Ruiz-Duenas, and A.T. Martinez (1996) Purification and catalytic properties of two manganese isoenzymes from *Pleurotus eryngii. Eur. J. Biochem.* **237**: 424–432.

Martinez, D., L.F. Larrondo, N. Putnam, et al. (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. *Nat. Biotechnol.* **22**: 695–700.

Martinez-Ingio, M.J., and B. Kurek (1997) Oxidative degradation of alkali wheat straw lignin by fungal peroxidase, manganese peroxidase and laccase: a comparative study. *Holzforschung* **51**: 643–548.

Marwaha, S.S., R. Grover, C. Prakash, and J.F. Kennedy (1998) Continuous bio-bleaching of black liquor from the pulp and paper industry using an immobilized cell system. *J. Chem. Technol. Biotechnol.* **73**: 292–296.

Mehna, A., P. Bajpai, and P.K. Bajpai (1995) Studies on decolorization of effluent from a small pulp mill utilizing agriresidues with *Trametes versicolor. Enzyme Microb. Technol.* **17**: 18–22.

Messerschmidt, A., ed. (1997) *Multi-copper Oxidases.* World Scientific, Singapore.

Messner, K., G. Ertler, G. Jaklin-Farcher, P. Boskovsky, U. Regensberger, and A. Blaha (1990) Treatment of bleach plant effluents by the MYCOPOR system. In: *Biotechnology in Pulp and Paper Manufacture: Applications and Fundamental Investigations*, T.K. Kirk and H.M. Chang, eds. Butterworth-Heinemann, Boston, pp. 245–251.

Mester, T., and J.A. Field (1998) Characterization of a novel manganese peroxidase–lignin peroxidase hybrid isozyme produced by *Bjerkandera* species strain BOS55 in the absence of manganese. *J. Biol. Chem.* **273**: 15412–15417.

Mester, T., E. de Jong, and J.A. Field (1995) Manganese regulation of veratryl alcohol in white rot fungi and its indirect effect on lignin peroxidase. *Appl. Environ. Microbiol.* **61**: 1881–1887.

Mester, T., H.J. Swarts, S. Romero i Sole, J.A. de Bont, and J.A. Field (1997) Stimulation of aryl metabolite production in the basidiomycete *Bjerkandera* sp. strain BOS55 with biosynthetic precursors and lignin degradation products. *Appl. Environ. Microbiol.* **63**: 1987–1994.

Michel, F.C., Jr., S.B. Dass, E.A. Grulke, and C.A. Reddy (1991) Role of manganese peroxidases and lignin proxidases of *Phanerochaete chrysosporium* in the decolorization of kraft bleach plant effluent. *Appl. Environ. Microbiol.* **57**: 2368–2375.

Modi, D.R., H. Chandra, and S.K. Garg (1998) Decolourization of bagasse-based paper mill effluent by the white-rot fungus *Trametes versicolor. Bioresour. Technol.* **66**: 79–81.

Moen, M.A., and K.E. Hammel (1994) Lipid peroxidation by the manganese peroxidase by *Phanerochaete chrysosporium* is the basis of phenanthrene oxidation by the intact fungus. *Appl. Environ. Microbiol.* **60**: 1956–1961.

Moreira, M., G. Feijoo, R. Sierra-Alverez, J. Lema, and J.A. Field (1997) Biobleaching of oxygen delignified kraft pulp by several white rot fungal strains. *J. Biotechnol.* **53**: 237–251.

Moukha, S.M., T.J. Dumonceaux, E. Record, and F.S. Archibald (1999) Cloning and analysis of *Pycnosporus cinnabarinus* cellobiose dehydrogenase. *Gene* **234**: 23–33.

Muheim, A., R. Waldner, M.S.A. Leisola, and A. Fiechter (1990) An extracellular aryl-alcohol oxidase from the white-rot fungus *Bjerkandera adusta*. *Enzyme Microb. Technol.* **12**: 204–209.

Nagarathnamma, R., and P. Bajpai (1999) Decolorization and detoxification of extraction-stage effluent from chlorine bleaching of kraft pulp by *Rhizopus oryzae*. *Appl. Environ. Microbiol.* **65**: 1078–1082.

Nagarathnamma, R., P. Bajpai, and P.K. Bajpai (1999) Studies on decolourization, degradation and detoxification of chlorinated lignin compounds in kraft bleaching effluents by *Ceriporiopsis subvermispora*. *Process Biochem.* **34**: 939–948.

Nie, G., N.S. Reading, and S.D. Aust (1998) Expression of the lignin peroxidase H2 gene from *Phanerochaete chrysosporium* in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **249**: 146–150.

Nilsson, T., G. Daniel, T.K. Kirk, and J.R. Obst (1989) Chemistry and microscopy of wood decay by some higher ascomycetes. *Holzforschung* **43**: 11–18.

Otterbein, L., E. Record, S. Longhi, M. Asther, and S. Moukha (2000) Molecular cloning of the cDNA laccase from *Pycnosporus cinnabarinus* I-937 and expression in *Pichia pastoris*. *Eur. J. Biochem.* **267**: 1–8.

Pacheco, M., and M.A. Santos (1996) Induction of micronuclei and nuclear abnormalities in the erythrocytes of *Anguilla anguilla* exposed to either cyclophosphamide or to bleached plant effluent. *Fresenius' Environ. Bull.* **5**: 746–751.

Paice, M.G., L. Jurasek, C. Ho, R. Bourbonnais, and S.F. Archibald (1989) Direct biological bleaching of hardwood kraft pulp with the fungus *Coriolus versicolor*. *TAPPI J.* **72**: 217–221.

Pallerla, S., and R.P. Chambers (1995a) Continuous decolorization and AOX reduction of bleach plant effluents by free and immobilized *Trametes versicolor*. *J. Environ. Sci. Health A* **30**: 423–437.

Pallerla, S., and R.P. Chambers (1995b) New urethane prepolymer immobilized fungal bioreactor for decolorization and dechlorination of kraft bleach effluents. *TAPPI J.* **79**: 155–161.

Pallerla, S., and R.P. Chambers (1997) Characterization of a Ca-alginate-immobilized *Trametes versicolor* bioreactor for decolorization and AOX reduction of paper mill effluents. *Bioresour. Technol.* **60**: 1–8.

Palmieri, G., P. Giardina, L. Marzullo, B. Desiderio, G. Nittii, R. Cannio, and G. Sannia (1993) Stability and activity of a phenol oxidase from the ligninolytic fungus *Pleurotus ostreatus*. *Appl. Microbiol. Biotechnol.* **39**: 632–636.

Palmieri, G., P. Giardina, C. Bianco, A. Scaloni, A. Capasso, and G. Sannia (2000) Copper induction of laccase isoenzymes in the lignolytic fungus *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* **66**: 920–924.

Park, J.S.B., P.M. Wood, M.J. Davies, B.C. Gilbert, and A.C. Whitwood (1997) A kinetic and ESR investigation of iron(II) oxalate oxidation by hydrogen peroxide and dioxygen as a source of hydroxyl radicals. *Free Radic. Res.* **27**: 447–458.

Perez, J., L. Saez, L. de la Rubia, and J. Martinez (1997) *Phanerochaete flavido-alba* ligninolytic activities and decolorization of partially bio-depurated paper mill wastes. *Water Res.* **31**: 495–502.

Perry, C.R., S.E. Matcham, D.A. Wood, and C.F. Thurston (1993) The structure of laccase protein and its synthesis by the commercial mushroom *Agaricus bisporus*. *J. Gen. Microbiol.* **139**: 171–178.

Perumal, K., K. Murugesan, and P.T. Kalaichelvan (2000) Influence of culture parameters on paper mill effluent decolourization by a white rot fungus *Ganoderma lucidum*. *Indian J. Exp. Biol.* **38**: 385–389.

Piontek, K., T. Glumoff, and K. Winterhalter (1993) Low pH crystal structure of glycosylated lignin peroxidase from *Phanerochaete chrysosporium* at 2.5 Å resolution. *FEBS Lett.* **315**: 119–124.

Poppius-Levlin, K., T. Tamminen, A. Kalliola, and T. Ohra-aho (2001) Characterization of residual lignins in pulps delignified by laccase/*N*-hydroxyacetanilide. In: *Oxidative Delignification Chemistry: Fundamentals and Catalysis*, D.S. Argyropoulos, ed. ACS Symposium Series 785. American Chemical Society, Washington, DC, pp. 358–372.

Poulos, T.L., S.L. Edwards, H. Wariishi, and M.H. Gold (1993) Crystallographic refinement of lignin peroxidase at 2 Å. *J. Biol. Chem.* **268**: 4429–4440.

Prasad, D.V., and T.W. Joyce (1991) Color removal from kraft bleach plant effluents by *Trichoderma* sp. *TAPPI J.* **74**: 165–169.

Prouty, A.L. (1990) Bench scale development and evaluation of fungal bioreactor for colour removal from bleach effluents. *Appl. Microbiol. Biotechnol.* **32**: 490–493.

Radoticacute, K., J.-K. Simicacute, M. Jeremicacute, and M. Trifunovicacute (1994) A study of lignin formation at the molecular level by scanning tunneling microscopy. *Biophys. J.* **66**: 1763–1767.

Raghukumar, C., D. Chandramohan, F.C. Michel, and C.A. Reddy (1996) Degradation of lignin and decolorization of paper mill bleach plant effluent (BPE) by marine fungi. *Biotechnol. Lett.* **18**: 105–106.

Raices, M., R. Montesino, J. Cremata, B. Garcia, et al. (2002) Cellobiose quinone oxidoreductase from the white rot fungus *Phanerochaete chrysosporium* is produced by intracellular proteolysis of cellobiose dehydrogenase. *Biochim. Biophys. Acta* **1576**: 15–22.

Ramaswamy, V. (1987) Biotechnology applications in waste utilization and pollution abatement. In: *Proceedings of the IPPTA Convention*, Indian Pulp and Paper Association, Saharanpur, India, pp. 1–14.

Regaldo, V., A. Rodriguez, F. Perestelo, A. Carnicero, G. De la Fuente, M.A. Falcon (1997) Lignin degradation and modification by the soil-inhabiting fungus *Fusarium proliferatum*. *Appl. Environ. Microbiol.* **63**: 3716–3718.

Regaldo, V., F. Perestelo, A. Rodriguez, A. Carnicero, F.J. Sosa, et al. (1999) Activated oxygen species and two extracellular enzymes: laccase and aryl-alcohol oxidase, novel for the lignin-degrading fungus *Fusarium proliferatum*. *Appl. Microbiol. Biotechnol.* **51**: 388–390.

Reid, I.D. (1991) Intermediates and products of synthetic lignin (dehydrogenative polymerizate) degradation by *Phlebia tremellosa*. *Appl. Environ. Microbiol.* **57**: 2834–2840.

Rodriguez, A., F. Perestelo, A. Carnicero, V. Regaldo, and R. Perez, et al. (1996a) Degradation of natural lignins and lignocellulosic substrates by soil inhabiting fungi imperfect. *FEMS Microbiol. Ecol.* **21**: 213–219.

Rodriguez, A., M.A. Falcon, A. Carnicero, F. Perestelo, G. De la Fuente, and J. Trojanowski (1996b) Laccase activities of *Penicillium chrysogenum* in relation to lignin degradation. *Appl. Microbiol. Biotechnol.* **45**: 399–403.

Rodriguez, J., A. Ferraz, R.F.P. Nogueira, I. Ferrer, E. Esposito, and N. Duran (1997) Lignin degradation by the ascomycete *Chrysonilia sitophila*. *Appl. Biochem. Biotechnol.* **62**: 233–242.

Roy, B.P., M.G. Paice, F.S. Archibald, S.K. Misra, and L. Misiak (1994) Creation of metal-complexing agents, reduction of manganese dioxide and promotion of manganese peroxide mediated Mn(III) production by cellobiose:quinone oxidoreductase from *Trametes versicolor*. *J. Biol. Chem.* **269**: 19745–19750.

Roy-Arcand, L., and F.S. Archibald (1991) Comparison and combination of ozone and fungal treatments of a kraft bleachery effluent. *TAPPI J.* **74**: 211–218.

Royer, G., M. Desrochers, L. Jurasek, D. Rouleau, and R.C. Mayer (1985) Batch and continuous decolorisation of bleached kraft effluent by a white-rot fungus. *J. Chem. Technol. Biotechnol. B* **35**: 14–22.

Royer, G., J. Yerushalmi, D. Rouleau, and M. Desrochers (1991) Continuous decolorization of bleached kraft effluents by *Coriolus versicolor* in the form of pellets. *J. Ind. Microbiol.* **7**: 269–278.

Ruiz-Duenas, F.J., M.J. Martinez, and A.T. Martinez (1999a) Molecular characterization of a novel peroxidase isolated from the ligninolytic fungus *Pleurotus eryngii*. *Mol. Microbiol.* **31**: 223–235.

Ruiz-Duenas, F.J., M.J. Martinez, and A.T. Martinez (1999b) Heterologous expression of *Pleurotus eryngii* peroxidase confirms its ability to oxidize Mn^{2+} and different aromatic substrates. *Appl. Environ. Microbiol.* **65**: 4705–4707.

Ruttimann, C., R. Vicuna, M.D. Mozuch, and T.K. Kirk (1991) Limited bacterial mineralization of fungal degradation intermediates from synthetic lignin. *Appl. Environ. Microbiol.* **57**: 3652–3655.

Ruttimann, C., E. Schwembeer, L. Salas, D. Cullen, and R. Vicuna (1992) Ligninolytic enzymes of the white-rot basidiomycetes *Phebia brevispora* and *Ceriporiopsis subvermispora*. *Biotechnol. Appl. Biochem.* **16**: 64–76.

Saloheimo, M., and M.-L. Niku-Paavola (1991) Heterologous production of a ligninolytic enzyme expression of the *Phlebia radiata* laccase gene in *Trichoderma reesei*. *Bio/Technol.* **9**: 987–990.

Saloheimo, M., V. Barajas, M.-L. Niku-Paavola, and J.K.C. Knowles (1989) A lignin peroxidase-encoding-cDNA from the white-rot fungus *Phlebia radiata*: characterization and expression in *Trichoderma reesei*. *Gene* **85**: 343–351.

Saparrat, M.C.N., F. Guillen, A.M. Arambarri, A.T. Martinez, and M.J. Martinez (2002) Induction, isolation, and characterization of two laccases from the white rot basidiomycete *Coriolopsis rigida*. *Appl. Environ. Microbiol.* **68**: 1534–1540.

Sastri, C.A. (1986) Colour removal from pulp and paper mill waste waters. *Indian J. Environ. Protect.* **6**: 105–113.

Schilling, B., R.M. Linden, U. Kupper, and K. Lerch (1992) Expression of *Neurospora crassa* laccase under the control of the copper-inducible metallothionein-promoter. *Curr. Genet.* **22**: 197–203.

Sealy, J.E., and A.J. Ragauskas (1998) Residual lignin studies of laccase-delignified kraft pulps. *Enzyme Microb. Technol.* **23**: 422–426.

Shimada, M., Y. Akamtsu, T. Tokimatsu, K. Mii, and T. Hattori (1997) Possible bio-chemical roles of oxalic acid as a low molecular weight compound involved in brown-rot and white-rot wood decays. *J. Biotechnol.* **53**: 103–113.

Singh, P., and I.S. Thakur (2005) Colour removal of anaerobically treated pulp and paper mill effluent by microorganisms in two steps bioreactor. *Bioresour. Technol.* **97**: 218–223.

Sollewijn Gelpke, M.D.S., M. Mayfield-Gambill, G.P. Lin Cereghino, and M.H. Gold (1999) Homologous expression of recombinant lignin peroxidase in *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **65**: 1670–1674.

Srebotnik, E., K.A. Jensen, Jr., S. Kawai, and K.E. Hammel (1997) Evidence that *Ceriporiopsis subvermispora* degrades nonphenolic lignin structures by a one-electron-oxidation mechanism. *Appl. Environ. Microbiol.* **63**: 4435–4440.

Stapleton, P.C., M.M. O'Brien, J. O'Callaghan, and A.D.W. Dobson (2004) Molecular cloning of the cellobiose dehydrogenase gene from *Trametes versicolor* and expression in *Pichia pastoris*. *Enzyme Microb. Technol.* **34**: 55–63.

Steffen, K., M. Hofrichter, and A. Hatakka (1999) Ligninolytic enzymes of litter-decaying basidiomycetes and their ability to degrade ^{14}C-labeled lignin. In: *Book of Abstracts of the Seventh International Fungal Biology Conference*, University of Groningen, Groningen, The Netherlands, 82 pp.

Steffen, K., M. Hofrichter, and A. Hatakka (2000) Mineralization of ^{14}C-labelled synthetic lignin and ligninolytic enzyme activities of litter-decomposing basidio-mycetous fungi. *Appl. Microbiol. Biotechnol.* **54**: 819–825.

Stewart, P., and D. Cullen (1999) Organization and differential regulation of a cluster of lignin peroxidase genes of *Phanerochaete chrysosporium*. *J. Bacteriol.* **181**: 3427–3432.

Stewart, P., R.E. Whitwam, P.J. Karsten, D. Cullen, and M. Tien (1996) Efficient expression of a *Phanerochaete chrysosporium* manganese peroxidase gene in *Aspergillus oryzae*. *Appl. Environ. Microbiol.* **62**: 860–864.

Streit, J.D., B.A. Prior, and S.G. Kilian (1987) Substrate utilization by yeast in a spent sulphite liquor permeate. *Water S.A.* (*Pretoria*) **13**: 145–150.

Subramanyam, P.V.R. (1990) Waste management in pulp and paper industry. *J. Indian Assoc. Environ. Manag.* **17**: 79–94.

Sumathi, S., and V. Phatak (1999) Fungal treatment of bagasse based pulp and paper mill wastes. *Environ. Technol.* **19**: 93–98.

Sundaramoorthy, M., K. Kishi, M.H. Gold, and T.L. Poulos (1994) The crystal structure of manganese peroxidase from *Phanerochaete chrysosporium* at 2.06-Å resolution. *J. Biol. Chem.* **269**: 32759–32767.

Sundaramoorthy, M., K. Kishi, M.H. Gold, and T.L. Poulos (1997) Crystal structures of substrate binding sites mutants of manganese peroxidase. *J. Biol. Chem.* **272**: 17574–17580.

Tanaka, H., S. Itakura, and A. Enoki (1999) Hydroxyl radical generation by an extracellular low-molecular-weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete *Trametes versicolor. J. Biotechnol.* **75**: 57–70.

Tanaka, H., S. Itakura, and A. Enoki (2000) Phenol oxidase activity and one-electron oxidation activity in wood degradation by soft-rot deuteromycetes. *Holzforschung* **54**: 463–468.

Taseli, B.K., and C.F. Gokcay (1999) Biological treatment of paper pulping effluents by using a fungal reactor. *Water Sci. Technol.* **40**: 93–99.

Taseli, B., C.F. Gokcay, and H. Taseli (2004) Upflow column reactor design for dechlorination of chlorinated pulping wastes by *Penicillium camemberti. J. Environ. Manag.* **72**: 175–179.

Tello, M., G. Corsini, L.F. Larrondo, L. Salas, S. Lobos, and R. Vicuna (2000) Characterization of three new manganese peroxidase genes from the ligninolytic basidiomycete *Ceriporiopsis subvermispora. Biochim. Biophys. Acta* **1490**: 137–144.

Temp, U., and C. Eggert (1999) Novel interaction between laccase and cellobiose dehydrogenase during pigment synthesis in the white rot fungus *Pycnosporus cinnabarinus. Appl. Environ. Microbiol.* **65**: 389–395.

ten Have, R., and M.C. Franssen (2001) On a revised mechanism of side product formation in the lignin peroxidase catalyzed oxidation of veratryl alcohol. *FEMS Lett.* **487**: 313–317.

ten Have, R., and J.M. Teunissen (2001) Oxidative mechanisms involved in lignin degradation by white-rot fungi. *Chem. Rev.* **101**: 3397–3413.

ten Have, R., S. Hartmans, P.J.M. Teunissen, and J.A. Field (1998) Purification and characterization of two lignin peroxidase isozymes produced by *Bjerkandera* sp. strain BOS55. *FEBS Lett.* **422**: 391–394.

Teunissen, P.J.M., and J.A. Field (1998) 2-Chloro-1,4-dimethoxybenzene as a mediator of lignin peroxidase catalyzed oxidations. *FEBS Lett.* **439**: 219–233.

Thakur, I.S. (2004) Screening and identification of microbial strains for removal of color and adsorbable organic halogens in pulp and paper mill effluent. *Process Biochem.* **39**: 1693–1699.

Thompson, D.N., B.R. Hames, C.A. Reddy, and H.E. Grethlein (1998) In vitro degradation of natural insoluble lignin in aqueous media by the extracellular peroxidases of *Phanerochaete chrysosporium. Biotechnol. Bioeng.* **57**: 704–717.

Tien, M., and T.K. Kirk (1983) Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium. Science* **221**: 661–663.

Tornberg, K., and S. Olsson (2002) Detection of hydroxyl radicals produced by wood-decomposing fungi. *FEMS Microbiol. Ecol.* **40**: 13–20.

Tuomela, M., M. Vikman, A. Hatakka, and M. Itavaara (2000) Biodegradation of lignin in a compost environment: a review. *Bioresour. Technol.* **72**: 169–183.

Tuomela, M., P. Olivanen, and A. Hatakka (2002) Degradation of synthetic ^{14}C-lignin by various white-rot fungi in soil. *Soil Biol. Biochem.* **34**: 1613–1620.

Tuor, U., H. Wariishi, H.E. Schoemaker, and M.H. Gold (1992) Oxidation of phenolic arylglycerol beta-aryl ether lignin model compounds by manganese peroxidase

from *Phanerochaete chrysosporium*: oxidative cleavage of an alpha-carbonyl model compound. *Biochemistry* **31**: 4986–4995.

Urzua, U., L. Larrondo, S. Lobos, J. Larrain, and R. Vicuna (1995) Oxidation reactions catalyzed by manganese peroxidase isoenzymes from *Cerioporiopsis subvermispora*. *FEMS Lett.* **371**: 132–136.

Valli, K., H. Wariishi, and M.H. Gold (1990) Oxidation of monomethoxylated aromatic compounds by lignin peroxidase: role of veratryl alcohol in lignin biodegradation. *Biochemistry* **29**: 8535–8539.

Vane, C.H. (2003) The molecular composition of lignin in spruce decayed by white-rot fungi (*Phanerochaete chrysosporium* and *Trametes versicolor*) using pyrolysis-GC-MS and thermochemolysis with tetramethylammonium hydroxide. *Int. Biodeterior. Biodegrad.* **51**: 67–75.

Varela, E., A.T. Martinez, and M.J. Martinez (1999) Molecular cloning of aryl-alcohol oxidase from *Pleurotus eryngii*, an enzyme involved in lignin degradation. *Biochem. J.* **341**: 113–117.

Varela, E., B. Bockle, B. Romero, A.T. Martinez, and M.J. Martinez (2000) Biochemical characterization, cDNA cloning and protein crystallization of aryl-alcohol oxidase from *Pleurotus pulmonarius*. *Biochim. Biophys. Acta* **1476**: 129–138.

Vares, T., and A. Hatakka (1997) Lignin-degrading activity and ligninolytic enzymes of different white-rot fungi: effects of manganese and malonate. *Can. J. Bot.* **75**: 61–71.

Vares, T., T.K. Lundell, and A. Hatakka (1992) Novel heme-containing enzyme possibly involved in lignin degradation by the white-rot fungus *Junghuhnia separabilima*. *FEMS Microbiol. Lett.* **99**: 53–58.

Vares, T., T.K. Lundell, and A. Hatakka (1993) Production of multiple lignin peroxidases by the white-rot fungus *Phlebia ochraceofulva*. *Enzyme Microb. Technol.* **15**: 664–669.

Vares, T., O. Niemenmaa, and A. Hatakka (1994) Secretion of ligninolytic enzymes and mineralization of ^{14}C-ring-labelled synthetic lignin by three *Phlebia tremellosa* strains. *Appl. Environ. Microbiol.* **60**: 569–575.

Vicuna, R. (1988) Bacterial degradation of lignin. *Enzyme Microb. Technol.* **10**: 646–655.

Vicuna, R., B. Gonzalez, D. Seelenfreund, C. Ruttimann, and L. Salas (1993) Ability of natural bacterial isolates to metabolize high and low molecular weight lignin-derived molecules. *J. Biotechnol.* **30**: 9–13.

Wahleithmer, J.A., F. Xu, K. Brown, S. Brown, et al. (1996) The identification and characterization of four laccase genes from the plant pathogenic fungus *Rhizoctonia solani*. *Curr. Genet.* **29**: 395–403.

Wariishi, H., L. Akaleswaran, and M.H. Gold (1988) Manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: spectral characteristics of oxidized states and catalytic cycle. *Biochemistry* **27**: 5365–5371.

Wariishi, H., K. Valli, and M.H. Gold (1989) Oxidative cleavage of a phenolic diarylpropane lignin model dimer by manganese peroxidase from *Phanerochaete chrysosporium*. *Biochemistry* **28**: 6017–6023.

Wariishi, H., K. Valli, and M.H. Gold (1991) In vitro depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Commun.* **176**: 269–275.

Wariishi, H., D. Sheng, and M.H. Gold (1994) Oxidation of ferrocytochrome *c* by lignin peroxidase. *Biochemistry* **33**: 5545–5552.

Watanabe, T., K. Koller, and K. Messner (1998) Copper-dependent depolymerization of lignin in the presence of fungal metabolite, pyridine. *J. Biotechnol.* **62**: 221–230.

Watanabe, T., H. Teranishi, Y. Honda, and M. Kuwahara (2002) A selective lignin-degrading *Ceriporiopsis subvermispora*, produces alkylitaconates that inhibit the production of a cellulolytic active oxygen species, hydroxyl radical in the presence of iron and H_2O_2. *Biochem. Biophys. Res. Commun.* **297**: 918–923.

Wu, J., Y.-Z. Xiao, and H.-Q. Yu (2005) Degradation of lignin in pulp mill waste-waters by white-rot fungi on biofilm. *Bioresour. Technol.* **96**: 1357–1363.

Yaver, D.S., and E.J. Golightly (1996) Cloning and characterization of three laccase genes from the white-rot basidiomycete *Trametes villosa*: genomic organization of the laccase gene family. *Gene* **181**: 95–102.

Yaver, D.S., F. Xu, E.J. Golightly, K.M. Brown, et al. (1996) Purification, characterization, molecular cloning, and expression of two laccase genes from the white-rot basidiomycete *Trametes villosa*. *Appl. Environ. Microbiol.* **62**: 834–841.

Yaver, D.S., M.C. Overjero, F. Xu, B.A. Nelson, K.M. Brown, et al. (1999) Molecular characterization of laccase genes from the basidiomycete *Coprinus cinereus* and heterologous expression of the laccase Lcc1. *Appl. Environ. Microbiol.* **65**: 4943–4948.

Yoshida, M., T. Ohiro, K. Igarashi, H. Nagasawa, and M. Samejima (2002) Molecular cloning and characterization of a cDNA encoding cellobiose dehydrogenase from the wood-rotting fungus *Grifola frondosa*. *FEMS Microbiol. Lett.* **217**: 225–230.

Zapanta, L.S., and M. Tien (1997) The roles of veratryl alcohol and oxalate in fungal lignin degradation. *J. Biotechnol.* **53**: 93–102.

Zhang, F.-M., J.S. Knapp, and K.N. Tapley (1999) Decolorization of cotton bleaching effluent with wood rotting fungus. *Water Res.* **33**: 919–928.

Zhao, J., and H.S. Kwan (1999) Characterization, molecular cloning, and differential expression analysis of laccase genes from the edible mushroom *Lentinula edodes*. *Appl. Environ. Microbiol.* **65**: 4908–4913.

Zimmerman, W. (1990) Degradation of lignin by bacteria. *J. Biotechnol.* **13**: 119–130.

10

FUNGAL DECOLORIZATION AND DEGRADATION OF DYES

10.1 INTRODUCTION

Large quantities of water and chemicals are used for the processing of textiles in the textile dye industries. These processes include desizing, scouring, bleaching, neutralizing, dyeing, printing, and finishing, leading to the generation of the dyed product and dye wastewater. Chemicals vary from inorganics to polymers to organic products. More than 100000 synthetic dyes exist commercially, with over 7×10^5 tons produced annually worldwide. An estimated 10 to 15% is directly lost in the wastewater, which carries a potential to cause environmental harm, including the formation of toxic amines under anaerobic conditions. Up to 50% of dyes are lost through hydrolysis in the reactive dye process, and the environmental fate is largely unknown. A problem of heavy metal ions also exists in wastewaters of metal-containing dyes.

Synthetic dyes are employed increasingly in the textile, paper, cosmetic, pharmaceutical, and food industries, due to high stability, wide variety of color, and good cost-effectiveness in synthesis compared to natural dyes. Dye color in industrial wastewaters is one of the most important environmental concerns in the textile industry. Water-soluble reactive dyes consist mainly of azo, anthraquinone, formazan, and phthalocyanine dyes. These are also used in printing industries, color photography, and as additives in petroleum products. The color must be removed from wastewaters before discharge to land or any water body. Even small amounts of dyes (less than 1 ppm) in water influence the aesthetic value in various water bodies. Removal of color is

given more consideration than removal of the soluble colorless organic materials that are responsible for the biochemical oxygen demand (BOD). Engineers have already established methods for the removal of BOD, but dyes are more difficult to remediate, due to the presence of complex aromatic compounds.

In recent years, interest in environmental control of dyes has increased, due to their possible toxicity and carcinogenicity. This is because many dyes are comprised of known carcinogens, such as benzidine and other aromatic compounds. The reduction of azo dyes in the intestinal environment, in anaerobic sediments, and decomposition to potential carcinogenic amines has already been established. Resistance of anthraquinone dyes due to fused aromatic compounds and greater color intensity of basic dyes and chromium-containing dyes, releasing chromium, contributes to a tendency to toxic behavior. Current knowledge of the long-term deleterious effects of dyes has not been completely assessed. Some disperse dyes also show a tendency to bioaccumulate in aquatic systems. Heavy metal ions accumulate in algae and wheat plants exposed by textile wastewaters (Srivastava and Prakash, 1991). The toxicity of azo dyes has been predicted by Brown and DeVito (1993). Certain dyes are also toxic to the single-cell green alga *Selenastrum capricornutum* and the fathead minnow *Pimephales promelas* (Hao et al., 2000).

10.2 CLASSIFICATION, STRUCTURE, AND COLOR MEASUREMENTS

The complex structure of dye compounds is described in the *Kirk–Othmer Encyclopedia* (ECT, 1977) and in *Colour for Textiles* (Ingamells, 1993). The *Color Index*, prepared by the Society of Dyers and Colourists (1976), reported more than 8000 chemical products related to the dyeing process. The *Color Index* (CI) number is used for dye classification. After documentation of the chemical structure of a dye, a five-digit number is assigned. The first word pertains to the dye classification, and the second word is the shade or hue of the dye. For example, Acid Violet 34 is classified as CI 61 710.

Based on the structure of the chromophoric group, Zollinger (1991) classified dyes into different categories, such as azo, anthraquinone, phthalocyanine, and triarylmethane (triphenylmethane) dyes. These are also called *reactive dyes.* Among the other classes of dyes, the reactive dyes are the only colorants that can bond covalently with fabrics. Twelve different chromatophores of dyes are recognized. Dyes are also classified into acid, basic, direct, disperse, mordant, reactive, sulfur, azoic, and vat types (Hao et al., 2000). Textile dyes are classified into three categories: cationic, anionic, and nonionic (Mishra and Tripathy, 1993). Anionic dyes include direct, acid, and reactive dyes; cationic, basic dyes; and nonionic, disperse dyes. Based on the mode of dyes or molecular structure, dyes may be classified as acid, direct, dispersant, and so on, which cannot be employed as a criterion for

environmental purposes. *Azo dyes* contain one or more functional groups, such as nitro-, carboxyl-, and sulfonic acid. Metal-complex dyes are known as *phthalocyanine dyes*. These dyes are characterized by at least one metal in their composition. *Anthraquinone dyes* contain more methyl groups. *Triphenylmethane dyes* are composed of three phenyl groups along with methyl groups. The molecular structures of azo, anthraquinone, phthalocyanine, triphenylmethane, and other dyes are well known in the literature.

Five methods have been established for the determination of color in samples (APHA, 1995). These include the visual comparison, spectrophotometric, tristimulus, ADMI (American Dye Manufacturer Institute) tristimulus, and alternate ADMI methods. Quantitative methods to determine the concentration of dyes include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and high-performance capillary electrophoresis. Other methods are HPLC and electrochemical detectors and cathode stripping voltammetry.

10.3 LEGISLATION AND REGULATIONS

Regulatory agencies are concerned with environmental and public health related to discharge of effluents from the textile and dyestuff industries and wastewater treatment systems to water bodies. Imposition of stringent environmental legislation in the United Kingdom, United States, and most developing countries has caused an increased problem for the textile and dye industries. State and federal agencies in the United States (McCurdy et al., 1992) require color limits of <200 ADMI units. The U.S. Environmental Protection Agency's toxic release inventory has calculated an annual discharge of approximately 2200 kg of four hazardous dyes to publicly owned treatment works (USEPA, 1993). In addition, the color standards in different states may vary within different localities, based on the receiving water quality. The Alabama dye waste discharge permit does not allow more than 50 ADMI units in the downstream color in the receiving river above the upstream color (Kennedy et al., 1992). In Virginia, color should not exceed 2000 ADMI units for effluent discharge into a municipal wastewater treatment plant (Ganesh et al., 1994).

During the past decade, color standards have been established in various parts of the world. In Germany, color standards for textile effluent are based on the absorbance measurements for three wavelengths and are calculated by a formula (Gahr et al., 1994). In Italy, the regulatory agencies require undetectable color when diluted 1:2 for certain receiving water bodies (Rozzi et al., 1999). The National Rivers Authority (UK Environmental Agency) requires "water-white" discharges in the textile industry, and even at present, many textile dye industries are experiencing problems in compliance. The absorbance values for the river quality of a filtered sample for seven wavelengths are established in the form of regulatory standards and consent levels

(Churchley, 1994). In the United Kingdom, color removal from textile effluent is regulated by the Environment Agency (EA) and the Scottish Environmental Protection Agency (SEPA) (Willmott et al., 1998). Denmark has forbidden the use of several azocolorants in textiles and toys due to potential formation of aromatic amines (DEPA, 1998). The new European Community (EC) regulations focus on the economic front of the discharge of dye wastewaters. In addition, European Council Directive 96/61/EC (Shaw, 1998) and the U.S. Pollution Prevention Act recommend end-of-pipe treatment to combat difficult-to-treat dye wastewaters. The Australian Dye Company and United Nations Environment Program (UNEP) are also working on cleaner production. In Taiwan, an ADMI standard of 550 at three wavelengths is necessary for discharge.

10.4 ALTERNATIVE DECOLORIZATION TREATMENT TECHNOLOGIES

In recent years, worldwide efforts have been employed to develop more effective color removal processes. However, no universal method is known for the treatment of dye wastewaters because of the complex and varied chemical structure of these compounds. Research in the past two decades has focused on the complexity of decolorization techniques of dye effluents. The dye concentrations of different classes in wastewaters have been recorded (Uygur, 1997). The characteristics of dyehouse wastewater, including alkalinity, COD, and BOD, are described by Kothandaraman et al. (1976). Several treatments are recognized related to decolorization of the textile or dye-containing wastewaters, and these are listed in Table 10.1. Various physical, chemical, electrochemical, photocatalytic, and biological processes, including bacterial bioreactors, are described along with possible mechanisms of action and various examples related to dye decolorization and degradation (Hao et al., 2000). Current treatment technologies for the remediation of dyes in textile effluent have also been illustrated with effective and cheaper alternatives for dye decolorization and removal on a large scale (Robinson et al., 2001a).

10.4.1 Physicochemical Methods

Physical, chemical, and electrochemical methods produce a large volume of sludge. These methods also exhibit noticeable differences in color removal, volume capability, duration of operation, and capital cost. Color in large volumes of wastewaters can be removed rapidly by membrane technology and ozone treatment, but the capital cost is high. Phthalocyanine dyes are quite stable in advanced oxidation processes, such as TiO_2/UV, Fenton, and photo-Fenton reagents (Arslan and Bacioglu, 1999). Ultrasonic approach for mineralization of dye is also demonstrated (Vinodgopal et al., 1998). Dyes can be removed more effectively by magnesium chloride than by alum and

TABLE 10.1 Methods for Decolorization of Dye-Containing Wastewaters

Physical	Chemical	Electrochemical	Photocatalytic	Biological
Adsorption	Coagulation	Electrooxidation	UV/H_2O_2	Aerated lagoon
Sedimentation	Precipitation	Electrocoagulation	UV/O_3	Anaerobic digestion
Flotation	Neutralization	Electroflotation	UV/TiO_2	Stabilization pond
Reverse osmosis	Flocculation			Activated sludge
Nano- and electrofiltration	Ion exchange			Trickling filter
	Oxidation			Bioaugmentation
Ionizing radiation	Reduction			Bacteria (aerobic or anaerobic)
Ultrasonic	Wet air oxidation			
	Chlorination			Fungi
	Ozonation			
	Fenton reagent			

polyaluminum chloride (Tan et al., 2000). Obviously, both physical and chemical methods have shortcomings and are expensive.

10.4.2 Biological Methods

10.4.2.1 Degradation by Bacteria. Presently, most of the textile industries are developing wastewater remediation technologies on-site in order to discharge below the criteria limits. A variety of bacterial bioreactors have been proposed to obtain an effective continuous anaerobic/aerobic treatment of dyes. These include (1) aerobic activated-sludge or rotating biofilm reactors (Shaul et al., 1991; Jiang and Bishop, 1994); (2) anaerobic fixed-film fluidized-bed reactors followed by aerobic suspended-bed activated sludge reactors (Seshadri et al., 1994; Fitzgerald and Bishop, 1995); (3) semicontinuous anaerobic reactors (Mass and Chaudhari, 2005); (4) anaerobic upflow fixed-bed columns with aerobic agitated tanks (An et al., 1996; Rajaguru et al., 2000); (5) aerobic–anaerobic sequential batch or continuous-flow reactors (Oxspring et al., 1986; Loyd et al., 1992; Ganesh et al., 1994); (6) combination of anaerobic and aerobic rotating drum reactors (Harmer and Bishop, 1992; Sosath and Libra, 1997); (7) anoxic–aerobic sequential bioreactors (Khehra et al., 2006); and (8) fed-batch column reactors (Kapdan and Oztekin, 2003).

Several genera and species of bacteria have been established for dye decolorization. Bacteria show inferior decolorizing capabilities for triphenylmethane than that for azo dyes. The decolorization capability is correlated to the molecular weight and octanol–water partition coefficient. Reverse decolorization also happens upon exposure of anaerobic degradation products to oxygen. Due to the specificity of bacteria, they cannot be employed in degradation of dye mixtures in wastewaters. Bacterial peroxidases, cometabolic reductive cleavage by aerobic bacteria as the sole source of carbon and energy, aerobic reductases, anaerobic reduction and its mechanism, and anaerobic/aerobic treatment of azo dyes have been summarized by Stolz (2001).

10.4.2.2 Degradation by Actinomycetes. A soil actinomycete, *Streptomyces chromofuscus*, exhibits less decolorization than the white-rot fungus *Phanerochaete chrysosporium* (Paszczynski et al., 1992). Several actinomycete strains decolorize the reactive dyes, including azo, anthraquinone, and phthalocyanine, through adsorption (Zhou and Zimmermann, 1993). *Pseudomonas luteola* completely degrades the reactive azo dyes (e.g., RBB, RP_2B, and V_2RP), whereas the azo bond is cleaved for the Red G dye (Hu, 1994). The degradation product of Crystal Violet by both actinomycetes is Michler's ketone (MK). Methyl Violet, Ethyl Violet, Basic Fuchsin, and Victoria Blue are also decolorized.

10.4.2.3 Degradation by Algae and Higher Plants. Azo dyes are degraded by an induced form of azoreductase by algae (Jinqi and Houtian, 1992).

Several species of *Chlorella* and *Oscillatoria* degrade azo dyes to aromatic amines and eventually, biotransform to simpler organic compounds or CO_2. Some species exhibit the ability to utilize azo dyes as the sole source of carbon and nitrogen. These algae can be added to stabilization ponds for the elimination of aromatic amines. The sulfonated anthraquinones are removed via uptake and transformation by free rhubarb (*Rheum palmatum*) cells grown in bioreactors after 20 days (Schwitzguebel and Vanek, 2003). It appears that certain enzymes of rhubarb involved in this transformation can be harnessed in the development of a biological process to treat wastewater from the dye and detergent industries.

10.5   FUNGAL DECOLORIZATION AND DEGRADATION OF DYES

During the past decade, fungi have been demonstrated to degrade various classes of dyes. It is a good alternative to replace or supplement present treatment processes. Fungal treatment of dyes is an economical and feasible method for decolorizing textile wastewaters. The state of the art on microbial dye decolorization and biodegradation also encompasses the role of fungi and white-rot fungi (Banat et al., 1996). Fungal decolorization and degradation of dyes and dye wastewater have been described by some researchers (Hao et al., 2000; Fu and Viraraghavan, 2001; Knapp et al., 2001). Fungal decolorization and degradation of different types of dyes are noted in Table 10.2.

10.5.1   Azo Dyes

Azo dyes constitute the most versatile and largest class of synthetic dyes employed commercially in the textile and food industries. More than 2000 different azo dyes are used to dye various materials, such as textile, leather, plastic, cosmetics, and food. These are characterized by the presence of one or more azo bonds (-N<=>N-) in association with one or more aromatic systems that may also carry sulfonic acid groups. These are also the most common class of dyes released into aquatic and terrestrial environments through the effluents, resulting in the contamination of rivers and groundwaters. Cationic dyes are more toxic, followed by anionic acid and direct dyes. Azo dyes used in degradation by *Phanerochaete chrysosporium* (Spadaro et al., 1992), except 4-phenylazophenol and 4-phenylazo-2-methoxyphenol, are considered toxic by the U.S. Environmental Protection Agency (Lowry and Lowry, 1988). *P. chrysosporium* also degrades textile dyes with bioaccessible groups such as guaiacol (2-methoxyphenol) and syringol (2,6-dimethoxyphenol) (Martins et al., 2001). Degradation of azo dyes by *P. chrysosporium* is enhanced by introducing a guaiacol substituent into the chemical structure of the dye, thus making it more amenable to

TABLE 10.2 Fungal Decolorization and Degradation of Dyes

Fungus	Dye Conc.	Medium	Color Removal (%)	Mechanism	Incubation (hours/days)	Reference
Phanerochaete chrysosporium	Orange II, 57µM Azure B, 16µM Congo Red, 76µM Tropaeolin O, 63µM	N-limited media	100 100 97 96	Biodegradation and adsorption	5d 1d 5d 2d	Cripps et al., 1990
P. chrysosporium	Congo Red, 500mg/l	N-limited culture	70	Adsorption, biodegradation, and lignin-degrading system	2d	Tatarko and Bumpus, 1998
Polyporus ostreiformis	Congo Red	N-limited culture	99	MnP and LiP	9d	Dey et al., 1994
P. chrysosporium	Reactofix Gold Yellow Navinon Blue	N-limited medium	73 71	Lignin-degrading system and adsorption	3d	Capalash and Sharma, 1992
Natural isolate of *Coriolus versicolor*	Acid Green 27, 0.5g/l Cu-phthalocyanine tetrasulfonic acid, 0.5g/l Indigo Carmine, 0.5g/l Neutral Red, 0.5g/l Acid Red 106, 0.5g/l Mordant Yellow, 0.5g/l Brilliant Yellow, 0.5g/l Reactive Red 4, 0.5g/l Orange II, 0.5g/l Brilliant Green, 0.5g/l	N-limited medium	100 99 100 91 100 84 99 90 100 100	Biodegradation and adsorption	7d	Knapp et al., 1995
Trametes versicolor	Hydrolyzed, 200mg/l Reactive Violet 5 Hydrolyzed, 200mg/l Reactive Blue 38	Liquid glucose medium	95 95	Adsorption and lignin-degrading system	6d 4d	Heinfling et al., 1997

TABLE 10.2 *Continued*

Fungus	Dye Conc.	Medium	Color Removal (%)	Mechanism	Incubation (hours/days)	Reference
Bjerkandera adusta	Hydrolyzed Reactive Violet 5, 200mg/l		95		6d	
	Hydrolyzed Reactive Blue 38, 200mg/l		95		4d	
P. chrysosporium	Acid Violet 7, 50mg/l	N-limited liquid medium	27	Adsorption and biodegradation	9d	Young and Yu, 1997
	Acid Blue 25, 50mg/l		55			
	Acid Black 24, 50mg/l		98			
	Reactive Black 5, 50mg/l		11			
	Reactive Blue, 50mg/l		76			
	Indigo Carmine, 50mg/l		30			
T. versicolor	Acid Violet 7, 50mg/l	N-limited liquid medium	12			
	Acid Blue 25, 50mg/l		69			
	Acid Black 24, 50mg/l		97			
	Reactive Black 5, 50mg/l		16			
	Reactive Blue, 50mg/l		91			
	Indigo Carmine, 50mg/l		93			
T. versicolor CNPR 8107	Remazol Blue RR, 60mg/l	Kirk's medium	96	Biodegradation and lignin-degrading system	6d	Toh et al., 2003
	Remazol Red RR, 60mg/l		96			
T. versicolor ATCC 20869	Remazol Blue RR, 60mg/l		98			
	Remazol Red RR, 60mg/l		85			
P. chrysosporium	Reactive Red 22, 120–140mg/l	C-limited medium N-limited medium	92	Lignin-degrading system	5h 30h	Wu et al., 1996
			100			
Aspergillus sojae B-10	Amaranth, 10mg/l	N-limited culture	98	Biodegradation and adsorption	5d	Ryu and Weon, 1992
	Sudan III, 10mg/l		97			
	Congo Red, 10mg/l		93			
Aspergillus foetidus	Remazol Red		>98	Adsorption	2d	Sumathi and Phatak, 1999
	Remazol Brown, 50mg/l		>98			

Organism	Dye, concentration	%	Medium	Mechanism	Time	Reference
	Remazol Dark Blue HR, 50 mg/l	>99				
	Remazol Brilliant Orange, 50 mg/l	68				
A. foetidus	Drimarene Red 50 mg/l	>99	Mineral salts medium	Adsorption	75h	Sumathi and Manju, 2000
	200 mg/l	>99			60h	
	Drimarene Navy Blue 50 mg/l	30				
	200 mg/l	30			100h	
P. chrysosporium	Amaranth, 50 mg/l	>98	Liquid media	Biodegradation and adsorption	8d	Chagas and Durrant, 2001
	New Coccine, 50 mg/l	95				
	Orange G, 50 mg/l	97				
Pleurotus sajor-caju	Amaranth, 50 mg/l	97				
	New Coccine, 50 mg/l	>96				
Pleurotus pulmonarius	Congo Red, 200 ppm	93	Glucose ammonium tartrate–corncob solid-state cultures	Biodegradation, adsorption, and laccase	6d	Tychanowicz et al., 2004
	Trypan Blue, 200 ppm	95				
	Amido Black, 200 ppm	89				
	Remazol Brilliant Blue R, 200 ppm	97				
	Methylene Blue, 200 ppm	57				
	Poly R-478, 200 ppm	55				
	Ethyl Violet, 200 ppm	88				
	Methyl Violet, 200 ppm	93				
	Methyl Green, 200 ppm	95				
	Brilliant Cresyl Blue, 200 ppm	90				
Irpex lacteus	Methyl Red, 150 µg/g	56	Low-nitrogen mineral medium	Lignin-degrading system	14d	Novotny et al., 2001
	Congo Red, 150 µg/g	58				
	Cu-Phthalocyanine, 150 µg/l	98				
	Bromophenol Blue, 150 µg/g	100				

TABLE 10.2 *Continued*

Fungus	Dye Conc.	Medium	Color Removal (%)	Mechanism	Incubation (hours/days)	Reference
I. lacteus	Reactive Black 5, 150 mg/l	Mineral medium	90	Biodegradation	10 d	Maximo and Costa-Ferreira, 2004
	Reactive Blue 19 (RBBR), 150 mg/l		>90		2 d	
P. chrysosporium	Cu-Phthalocyanine Remazol Turquoise Blue, 200 mg/l	Mineral salts medium	100		7 d	Conneely et al., 1999
Phlebia tremellosa	Remazol Turquoise Blue, 200 mg/l	Modified basal medium	>97		14 d	Kirby et al., 2000
Coriolus versicolor	Everzol Turquoise Blue G	Medium III, containing glucose		Biodegradation		Kapdan et al., 2000
	100–500 mg/l		100		6 d	
	700–1200 mg/l		80		9 d	
C. versicolor f. *antarcticus*	Xylidine, 24 mg/l	Liquid medium	28	Biodegradation and laccase	1 h	Levin et al., 2004
	Poly R-478, 75 mg/l		30			
	RBBR, 9 mg/l		43			
	Malachite Green, 6 mg/l		88			
	Indigo Carmine, 23 mg/l		98			
Trametes trogii	Anthraquinone Blue	Glucose-asparagine and malt extract/glucose	88	Laccase	4 h	Levin et al., 2001
Penicillium ATCC 74414 isolate	Poly R-478, 0.01%	HMS medium	100	Adsorption and biodegradation	4 d	Zheng et al., 1999
		PDB medium	100		9 d	
	Poly S-119, 0.01%	HMS and PDB media	100		4 d	
		SHK medium	100		4 d	
Geotrichum sp.	Reactive Black 5, 100 mg/l	N-limited medium	>99	Biodegradation, MnP, and laccase	10 d	Maximo et al., 2003
	Reactive Red 158, 100 mg/l		>99		20 d	
	Reactive Yellow 27, 100 mg/l		>99		20 d	

Organism	Dye	Medium	%	Mechanism	Time	Reference
Aspergillus niger *A. ochraceus* *A. parasiticus* *Marasmiellus troyanus*	Poly R-478, 0.02%	Sabouraud dextrose medium and then transfer to water	100 100 100 57	Adsorption	17 d	Wunch et al., 1997
Pleurotus sapidus			54			
P. ostreatus			54			
P. chrysosporium *T. versicolor* *Funalia trogii* *Laetiporus sulphureus*	Crystal violet, 50 µg/ml	Liquid medium	62 92 82 86	Biodegradation	3 d	Yesilada, 1995
Funalia trogii (pellets)	Astrazone Blue Astrazone Black Astrazone Red	Distilled water	92 94 97	Biodegradation	24h	Yesilada et al., 2003
P. chrysosporium (pellets)	Astrazone Blue Astrazone Black Astrazone Red		97 95 99			
C. versicolor (pellets)	Astrazone Blue Astrazone Black Astrazone Red		92 89 98			
Strain Wd1	Crystal Violet Brilliant Green	Liquid medium with Mn(II), H_2O_2, and glucose	36 62		2 d	Tayal et al., 1999
Strain I6	Crystal Violet Brilliant Green each 12ppm		44 46			
Cyathus bulleri	Crystal Violet, 1mg/ml Bromophenol Blue, 1mg/ml Malachite Green, 1mg/ml	Maltose-mineral medium	96 100 100	Laccase	3 d	Vasdev et al., 1995

TABLE 10.2 Continued

Fungus	Dye Conc.	Medium	Color Removal (%)	Mechanism	Incubation (hours/days)	Reference
Ganoderma (10 species)	Orange II, 100 mg/l 10B Blue, 100 mg/l RS Red, 100 mg/l	Potato dextrose broth culture	28–77 68–94 80–97	Adsorption	2 d	Mou et al., 1991
Myrothecium (9 species)	Orange II, 100 mg/l 10B Blue, 100 mg/l RS Red, 100 mg/l		25–91 59–99 81–99	Adsorption	2 d	
Thelephora sp.	Orange G, 50 μM Congo Red, 50 μM Amido Black 10B, 25 μM	C-limited medium	33 mg/g 97 mg/g 98.8 mg/g	Biodegradation	9 d 8 d 24 d	Selvam et al., 2003
Dichomitus squalens	Orange G, 500 mg/l Remazol Brilliant Blue R, 500 mg/l	N-limited Kirk, static N-rich Kirk, static	95 91	Laccase and MnP Laccase and MnP	14 d	Eichlerova et al., 2005
Ischnoderma resinosum	Orange G, 500 mg/l Remazol Brilliant Blue R, 500 mg/l	N-rich Kirk, static N-rich Kirk, static	95 99	Laccase MnP		
Pleurotus calyptratus	Orange G, 500 mg/l Remazol Brilliant Blue R, 500 mg/l	N-limited Kirk, static N-limited Kirk, static	91 78	Laccase MnP		
P. ostreatus	Remazol Brilliant Blue R, 50 μM	PDY broth	100	Laccase	9 d	Palmieri et al., 2005
P. chrysosporium	Five direct dyes, each 120 mg/l	N-limited medium	100	Biodegradation, adsorption, and MnP	15 d	Pazarlioglu et al., 2005

degradation by lignin-degrading enzymes (Paszczynski et al., 1991). The fungal degradation of azo dyes has been reviewed by Bumpus (1995) and Stolz (2001).

Congo dye is tightly adsorbed to mycelial pellets of *P. chrysosporium* in both ligninolytic and nonligninolytic cultures, but the dye is substantially degraded in ligninolytic cultures (Tatarko and Bumpus, 1998). The fruiting bodies of basidiomycete fungi growing on rotting wood decolorize a wide range of structurally different synthetic dyes (Knapp et al., 1995). Differences in decolorization of dyes may be attributed to steric factors, electron distribution, and charge density. Biodegradation is enhanced due to very specific structural changes in the dye molecules. Under certain conditions, incomplete decolorization depends on the chemical structure of dye molecules. Of 26 white-rot fungi, 10 strains decolorize all structurally different dyes and produce laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP) on solid medium (Levin et al., 2004). Pretreatment of *Aspergillus niger* dead biomass with $NaHCO_3$ removes nearly 90% of Congo Red in 42 hours at pH 6 (Fu and Viraraghavan, 2002). Biosorption of Congo Red follows the Lagergren first-order (Ho et al., 1996) and pseudo-second-order rate equations and the Radke–Prausnitz model.

Bjerkandera adusta reduced the EC_{20} values from 72 to 10 after 7 days during transformation of Hydrolyzed Reactive Violet 5 (HRV 5) (Heinfling et al., 1997). Strict secondary metabolism in strain *Trametes versicolor* CNPR 8107 was not necessary to produce ligninolytic enzymes (Toh et al., 2003). Low levels of MnP and laccase were detected after the completion of most of the decolorization of Reactive Black 5 by *Irpex lacteus* (Maximo and Costa-Ferreira, 2004). MnP, Mn-independent peroxidase (MIP), and laccase of *Geotrichum* sp. are involved in the transformation of Reactive Black 5 (Maximo et al., 2003). Additional enzymes or factors appear to be involved in the decolorization of Reactive Red 158 and Reactive Yellow 27. *Aspergillus foetidus* shows similar yields in the presence or absence of Drimarene dyes, implying tolerance in the presence of dyes and for growth (Sumathi and Manju, 2000). *P. chrysosporium* cultures produce the highest activities of MnP and β-glucosidase and thus incline towards the involvement of these enzymes in the degradation of these dyes (Chagas and Durrant, 2001). *Pleurotus sajor-caju* produces laccase active toward *o*-dianisidine, and glucose-1-oxidase may participate in the degradation process. Decolorization of Congo Red coincides with the formation of MnP of *I. lacteus* in a low-nitrogen mineral medium (Novotny et al., 2001). Crude exoenzyme MnP preparations from *P. chrysosporium* decolorize Direct Green 6, Chrysophenine, and Congo Red by 25%, 31%, and 33%, respectively (Pazarlioglu et al., 2005). Pellets of *Funalia trogii* in repeated-batch mode can be used for the decolorization of astrazone dyes within 24 hours (Yesilada et al., 2003). During solid-state fermentation, decolorization of structurally different dyes is related to the high laccase activity of *Pleurotus pulmonarius* (Tychanowicz et al., 2004).

Little is known about the fate of effluents from the chemical manufacturing industries. Highly colored diazo-linked chromophore in the effluent is present for the manufacture of nitrated stilbene–sulfonic acids (NSSA). The effluent also contains high levels of sodium chloride and sodium sulfate. Diazo-linked chromophore industrial effluent is decolorized by white-rot fungi (Knapp and Newby, 1999). The rates of decolorization are similar for all fungi in both salted and unsalted portions of the effluent. Such results have a potential for use in treating chemical industry wastes.

10.5.2   Phthalocyanine Dyes

Phthalocyanine dyes are widely used in the textile and dyestuff industries, but little is known about the decolorization and biodegradation of these compounds by microorganisms. *B. adusta* and *T. versicolor* reduced nickel about 30% at an initial Hydrolyzed Reactive Blue 38 (HRB 38) concentration of 200 mg/l (Heinfling et al., 1997). During transformation, these fungi show significant reduction in the toxicity of HRB 38 to *Vibrio fischeri*. After 7 days, the EC_{50} values were reduced to 9 (*B. adusta*) and 8 (*T. versicolor*) from 37. Five out of seven fungal isolates decolorized above 90% of Cu-phthalocyanine tetrasulfonic acid tetrasodium salt (Knapp et al., 1995). *I. lacteus* attained 98% decolorization of copper-phthalocyanine (CuPc) and showed the presence of MnP in liquid media (Novotny et al., 2001). Significant amounts of LiP or MnP activity were not detected during the decolorization of CuPc by *P. chrysosporium* PC671 (Conneely et al., 1999). This suggests little or no correlation between the production of ligninolytic enzymes and decolorization of this dye.

10.5.3   Anthraquinone Dyes

Both tropho- and idiophasic cultures of *Trametes trogii* BAFC 463 produce high levels of laccase activity under all conditions and may be related to decolorization of Anthraquinone Blue (Levin et al., 2001). *T. versicolor* degrades Acid Green 27 faster by extracellular than by intracellular enzymes (Wang and Yu, 1998). Five species of white-rot fungi decolorize Remazol Brilliant Blue R (Swamy and Ramsay, 1999a). Of 115 strains, 16 strains showed fast decolorization of Basic Blue 22 within 5 to 8 days (Jarosz-Wilkolazka et al., 2002). Two mitosporic fungi, *Pestalotia* sp. and *Tolypocladium* sp., produced an extracellular oxidative system and decolorized the dye within 11 days. *Flavodon flavus* decolorized several synthetic dyes (i.e., Azure B, Brilliant Green, Congo Red, Crystal Violet, and Remazol Brilliant Blue R) in a low-nitrogen medium (Raghukumar, 2000). *Pleurotus pulmonarius* decolorized 97% of Remazol Brilliant Blue R in corncob solid-state fermentation after 6 days (Tychanowicz et al., 2004).

10.5.4 Heterocyclic Dyes

Little is known about color removal of heterocyclic dyes. This may be due to the fact that these dyes are not used extensively in the textile industries. *P. chrysosporium* completely decolorized Azure B in a nitrogen-limited medium and 60% in a nitrogen-sufficient medium (Cripps et al., 1990). The non-ligninolytic medium revealed 14% of Azure B adsorbed to the fungal mycelia. Three isolates of fungi removed more than 95% of the color of Neutral Red (Knapp et al., 1995). *I. lacteus* decolorized Fluorescein and Methylene Blue completely on solid media (Novotny et al., 2001). Partial decolorization of Methylene Blue occurs due to *Pleurotus pulmonarius* in solid-state cultures (Tychanowicz et al., 2004).

10.5.5 Indigo Dyes

All fungal isolates of wood-rotting fungi remove about 99% of the color of Indigo Carmine, as evident by drastic spectral changes (Knapp et al., 1995). *T. versicolor* caused faster biodegradation of Indigo Carmine by intracellular enzymes or living mycelium containing intercellular enzymes (Wang and Yu, 1998). Decolorization was initiated in a few hours and *P. chrysosporium*, *Pycnosporus sanguineus*, *Pleurotus sajor-caju*, and *Phellinus gilvus* remove 75 to 100% of Indigo dye, respectively, after 4 days (Balan and Monteiro, 2001).

10.5.6 Polymeric Dyes

Not much work has been performed on the fungal degradation of polymeric dyes. Again, this may be because they are used comparatively less in the textile industries. *P. chrysosporium* decolorized three different polymeric dyes—Polymeric B-411, Polymeric R-481, and Polymeric Y-606—due to secondary metabolism (Glenn and Gold, 1983). *Trametes pocas*, *T. cingulata*, *T. versicolor*, and isolate DSPM95 decolorized Poly R-478 extensively and revealed high MnP activities, respectively (Tekere et al., 2001). Isolate DSPM95 and *T. versicolor* also showed high laccase activity and may be correlated with high dye decolorization. Of 17 strains of filamentous fungi belonging to 13 different species, living and dead mycelia of three species of *Aspergillus* showed 100% decolorization of Poly R-478 after 17 days (Wunch et al., 1997). The extracellular filtrate of *Laetiporus sulphureus* removed 68% of the color. Complete decolorization of Poly R-478 occurred in the presence of 0 and 5% acetone by *Bjerkandera* sp. strain BOS55 (Field et al., 1995).

10.5.7 Triphenylmethane Dyes

Triphenylmethane dyes are employed extensively in various industries due to their versatility. Numerous applications of triphenylmethane dyes are well

known in the textile industries, such as dying cotton, nylon, silk, and wool. These dyes are also used in the paper and leather industries, and many are used as biological stains and in veterinary medicine. They are also used in coloring plastics, fats, oils, and waxes and have applications in the cosmetics and food industries. Some triphenylmethane dyes are used extensively in medicine as dermatological agents. The biodegradation of triphenylmethane dyes by fungi and other organisms has been described by Azmi et al. (1998). Ligninolytic and nonligninolytic cultures of *P. chrysosporium* were found responsible for the degradation of several triphenylmethane dyes (Bumpus and Brock, 1988). Of six isolates, two strains of white-rot fungi, Wd1 and Id, enhanced the degradation of Crystal Violet and Brilliant Green to 36 and 62% and 44 and 46%, respectively, in the presence of Mn(II), H_2O_2, and glucose (Tayal et al., 1999). This indicates that peroxidases are involved in the degradation. Decolorization of dyes appears to be related to high titers of laccase activity in *Pleurotus pulmonarius* (Tychanowicz et al., 2004). Of three bird's nest fungi, *Cyathus bulleri* is the most efficient in decolorization of triphenylamine dyes, coinciding with the maximum activity of laccase (Vasdev et al., 1995). Four of seven isolates of white-rot fungi decolorized Brilliant Green completely (Knapp et al., 1995).

10.6   YEAST DECOLORIZATION AND DEGRADATION OF DYES

Little is known of dye degradation and color removal by yeasts. Table 10.3 indicates the decolorization and degradation of dyes by yeasts. The two oxidative yeasts *Rhodotorula* sp. and *R. rubra* degraded Crystal Violet completely in liquid broth after 4 days (Kwasniewska, 1985). The azo dyes can be eliminated from industrial effluents using yeast biomass (Angelis and Rodrigues, 1987). The percent color removal was reduced with an increasing initial concentration of azo dye effluents. A strain of yeast, *Candida zeylanoides*, degraded a number of simple azo dyes in liquid aerated batch bioreactors (Martins et al., 1999). Ignoring the differences in incubation periods, color loss using the Biolab batch bioreactor correlates well with that using shaken cultures. Metanilic acid is the reduction product (aminobenzenesulfonate) for azo dyes I and III, and sulfanilic acid for azo dyes II and IV by *Candida zeylanoides* (Ramalho et al., 2002). Other reduction products are 1-amino-2-naphthol and *N,N*-dimethyl-*para*-phenylenediamine, which are unstable in aerated solutions. This indicates that the yeast cleaves the reduction of azo bonds, producing amines. This behavior is also known in decolorizing anaerobic or facultative aerobic bacteria. *Saccharomyces cerevisiae* is a good candidate for dye removal in molasses media (Aksu, 2003). Biosorption of textile dyes occurs by biomass derived from the thermotolerant ethanol-producing yeast *Kluyveromyces marxianus* IMB3 (Bustard et al., 1998). Actively growing *K. marxianus* IMB3 yeast cells under aerobic conditions almost completely decolorized Remazol Black B within 24 hours at a pH between 3.5 and 5.0

TABLE 10.3 Yeast Decolorization and Degradation of Dyes

Yeast	Dye Conc.	Medium	Color Removal (%)	Mechanism	Incubation (hours/days)	Reference
Rhodotorula sp.	Crystal Violet, 10ppm	Liquid broth medium	>99	Unknown enzymes	4d	Kwasniewska, 1985
Rhodotorula rubra			>99			
Candida sp. strain	Procyon Black SPL, 100µg/l; Procyon Blue MX2G, 100µg/l; Procyon Red HE7B, 100µg/l; Procyon Orange HER, 100µg/l	Molasse medium	>93; >96; >98; >96	Adsorption	2h	Angelis and Rodrigues, 1987
Kluyveromyces marxianus	Remazol Black B, 25–200mg/l	Glucose mineral medium	>94	Adsorption	1d	Meehan et al., 2000
Candida zeylanoides	Azo dyes	Glucose-containing medium	46–67	Adsorption and biodegradation	22h	Martins et al., 1999
C. zeylanoides	Azo dyes I and II, 0.2mM; Azo dyes III and IV, 0.2mM	Normal decolorization medium	100; 15	Biodegradation	40h	Ramalho et al., 2002
C. guilliermondii	Remazol Blue, 200mg/l	Dye synthetic solution	41	Adsorption	15m	Aksu and Donmez, 2003
C. membranaefaciens			37			
C. lipolytica			37			
C. tropicalis			35			
Candida sp.			26			
Pseudozyma rugulosa Y-48	Reactive Brilliant Red, 50mg/l; Acid Mordant Light Blue, 50mg/l	Yeast-extract-peptone-dextrose (YEPD) medium	99; 89	Biodegradation	24h	Yu and Wen, 2005
Candida krusei G-1	Reactive Brilliant Red, 50mg/l; Acid Mordant Light Blue, 50mg/l		98; 93			

(Meehan et al., 2000). Temperature has a significant effect on decolorization, and an optimum temperature is 37°C. Magnetically modified brewer's yeast, *Saccharomyces cerevisiae* subsp. *uvarum* cells, efficiently adsorbs several water-soluble dyes (Safarikova et al., 2005).

10.7   WHITE-ROT FUNGAL DECOLORIZATION AND DEGRADATION OF DYES

The role of the white-rot fungus, *Phanerochaete chrysosporium* in the degradation of polymeric dyes was first established by Glenn and Gold (1983). Since then, several papers have appeared on the use of *P. chrysosporium* in the decolorization and degradation of dyes. In addition, other white-rot fungi (e.g., *Trametes versicolor*, *Pleurotus ostreatus*, and *Bjerkandera* sp.) have also been found to be efficient in degrading dyes. Several reports indicate the superiority of species of *Trametes* and *Bjerkandera* over *P. chrysosporium* in the rate and extent of decolorization of different dyes. A process has been patented for decolorizing dye effluent using white-rot fungi to absorb, degrade, and remove the color of effluent (Shen et al., 1992). The role of many white-rot fungi in dye decolorization and degradation was presented in the foregoing paragraphs. Based on current knowledge, it appears that about 50% of our knowledge of dye decolorization and degradation comes from white-rot fungi.

A fungal and chemical treatment using the sequence *Phanerochaete chrysosporium*–ozone suggests good decolorization, total phenols, and molecular mass reductions in textile effluent (Kunz et al., 2001). Decolorization was mainly through adsorption until day 6 and was related to the LiP activity between days 6 and 9. The toxicity of the textile effluent was reduced using *Scenedesmus subspicatus* and *Escherichia coli*, showing no inhibition at the end of the treatment.

10.8   MECHANISMS OF FUNGAL DECOLORIZATION AND DEGRADATION OF DYES

Colored textile wastewater contains a mixture of different aromatic dyes that are difficult to degrade. A wide range of pH and high salt concentrations of chlorides add more problems. Several synthetic dyes are resistant to microbial degradation and cannot be removed easily in conventional wastewater treatment systems. Shortcomings of both physicochemical and biological methods limit their large-scale applications in dye decolorization. Great interest is shown in a number of fungi, especially the versatile white-rot fungi, which have been explored to metabolize a wide range of recalcitrant organic pollutants. Possible mechanisms of fungal decolorization and degradation of dyes are listed in Table 10.4.

TABLE 10.4   Mechanisms of Fungal Dye Decolorization and Degradation

Adsorption
Biodegradation
Adsorption and biodegradation
Mineralization
Enzymatic degradation
Utilization as a carbon source

A variety of synthetic dyes have been found to be decolorized and degraded by fungi. Extracellular enzymes such as LiP and MnP are involved in the biodegradation of dyes as well as in color removal. The extent of dye removal depends on the degree of dye complexity, nitrogen limitation/availability, and the enzymatic activity in the medium. Increases in LiP and MnP enhanced decolorization under nitrogen-limited conditions. Higher rates of decolorization were achieved when the strains were cultivated in nitrogen-rich media, while no or slower decolorization occurred with strains cultivated in low-nitrogen media (Chao and Lee, 1994). Laccases are another group of enzymes involved in the biodegradation of dyes. Veratryl alcohol (VA) also appears to enhance ligninolytic activity and links to decolorization activity.

In addition to enzymatic degradation by white-rot fungi, the color of aqueous dyes can be removed through both adsorption and degradation by fungi. Adsorption has been called the mechanism of decolorization of a wide range of dye wastewater by several species of *Myrothecium* and *Ganoderma* (Mou et al., 1991). The process is insensitive to the variations in pH and high salt concentrations and is not bound by the particular mycelium of several fungal species and genera. A dye adsorption mechanism can be exploited in the treatment process for the removal and recovery of dyes from wastewater.

Myrothecium verrucaria binds strongly to azo dyes for rapid decolorization (Brahimi-Horn et al., 1992). Dyes bound to fungal biomass can be recovered by sonification or Triton X or extraction with methanol. The decolorization capability of *P. chrysosporium* and other decolorizing fungi and of *Candida* sp. related to dye concentration, percent removal/time, and possible mechanism has been discussed by Banat et al. (1996). Astrazon Red FBL decolorizes through adsorption by *Funalia trogii* pellets at the initial stage, followed by decolorization through microbial metabolism (Yesilada et al., 2002). Decolorization of Acid Dye 183 by three fungi (Jarosz-Wilkolazka et al., 2002) and of Amaranth, New Coccine, and Orange G by *P. chrysosporium* and *Pleurotus sajor-caju* (Chagas and Durrant, 2001) may be attributed to either adsorption on the fungal biomass or degradation.

The fate of adsorbed dye can be classified into three categories: (1) binding with the hyphal structures, (2) physical desorption to the solution, and (3) enzymatic degradation by living hyphal structures (Wang and Yu, 1998). The structure of individual dyes contributes to the adsorption ability and affinity

to fungal hyphae. The nonspecific biouptake process of adsorption is rapid and noncatholic to dye structures and adaptable to diverse types of textile wastewater. Physical desorption and enzymatic degradation can regenerate dye-saturated mycelium for the next adsorption. The fastest regeneration is obtained by the living mycelium plus extracellular enzymes. This mechanism is indicative of sequential adsorption and degradation of dyes by living fungal mycelium for the continuous treatment of effluent by *Trametes versicolor.* An interior region of rapidly settling spherical biomass pellets of *Aspergillus foetidus* shows strong bioadsorption rather than biotransformation of dyes (Sumathi and Manju, 2000). The presence of carboxylic and amino groups in Rhodamine B contributes to lower sorption using dead macrofungi, *Fomes fomentarius* and *Phellinus igniarius* (Maurya et al., 2006).

Mineralization of dyes rarely occurs during degradation by fungi. Mineralization (23 to 48%) of certain substituted radiolabeled azo dyes was reached after 12 days of addition using *P. chrysosporium* (Spadaro et al., 1992). Higher mineralization occurs in dyes containing aromatic rings substituents, such as amino, acetamido, hydroxy, or nitro functions than unsubstituted rings. Most dyes degrade more extensively under nitrogen-limiting conditions than under nitrogen-sufficient conditions.

During the last decade, there have been reports on the utilization of dyes as the sole source of carbon by fungi. These fungi do not contribute to the process of dye decolorization (Marchant et al., 1994; Nigam et al., 1995a,b). Certain bonds of dyes are cleaved in order to utilize them as carbon sources, but the chromophore is not affected. This type of behavior is prevalent when dye-degraders and dye-decolorizers are present in a consortium of microorganisms. The mutual relationship of dye-degraders and dye-decolorizers can lead to faster or complete degradation of dyes.

10.9   METABOLIC PRODUCTS AND PATHWAYS

Little is known on the formation of metabolic products and elucidation of pathways of dye degradation by fungi. However, metabolic products due to dye degradation by fungi are noted in Table 10.5. Three products appear during the sequential N-demethylation of Crystal Violet by *P. chrysosporium* (Bumpus and Brock, 1988). Two additional unidentified colored Crystal Violet metabolites were also found as a result of continued degradation, and ultimately, Crystal Violet degrades to a colorless product. Demethylation of Crystal Violet is also expressed with a H_2O_2-generating system in the extracellular fluid. Brilliant Green, Ethyl Violet, and Malachite Green contain *N*-alkyl groups, and thus initial oxidation of these dyes proceeds via N-demethylation in a manner similar to Crystal Violet. Bromophenol Blue, Cresol Red, and Pararosaniline contain no alkyl groups, and therefore oxidation of these dyes involves a mechanism different from that of Crystal Violet. This indicates the presence of LiP, which is nonspecific in

TABLE 10.5 Fungal Decolorization and Degradation of Dyes and Metabolic Products

Fungus	Dye Conc.	Medium	Color Removal (%)	Metabolic Products	Incubation (hours/days)	Reference
Phanerochaete chrysosporium	Crystal Violet 12.3 μM	N-limited culture	100	N,N,N',N',N''-penta-, N,N,N',N''-tetra-, and N,N',N''-trimethylpararosaniline	24 h	Bumpus and Brock, 1988
P. chrysosporium	Azure B, 16 μM Tropaeolin O, 63 μM Congo Red, 76 μM	N-limited and N-sufficient cultures N-sufficient cultures		Colored metabolites Metabolite	5 d	Cripps et al., 1990
P. chrysosporium	Disperse Yellow 3, 1 μM Naphthol analog, 1 μM	Stationary whole cultures	>99	Acetanilide (25%) Acetanilide (21%)	2 d	Spadro and Renganathan, 1994
P. chrysosporium	Remazol Turquoise Blue, 200 mg/l	Mineral salts medium	100	One metabolite	7 d	Conneely et al., 1999
Phlebia tremellosa	Remazol Black, 200 mg/l	Modified basal medium	>99	One metabolite	14 d	Kirby et al., 2000
Bjerkandera adusta	Reactive Blue 15, 200 mg/l Reactive Blue 38, 200 mg/l	Glucose ammonia solution	100	3- and 4-Sulfophthalimides 3- and 4-Sulfophthalimides	5 d	Heinfling-Weidtmann et al., 2001
Cunninghamella elegans	Malachite Green, 81 μM	Culture medium	85	Leucomalachite Green, N-demethylated and N-oxidized metabolites	1 d	Cha et al., 2001
P. chrysosporium	Rose Bengal, 40 μM	Liquid culture	99	One metabolite	5 h	Gogna et al., 1992
P. chrysosporium *Pycnosporus sanguineus*	Indigo textile, 0.02% (v/v)	Minimum medium MM	75 91	One metabolite	4 d	Balan and Monteiro, 2001
Pleurotus sajor-caju			94			
Phellinus gilvus			100			
Trametes versicolor	*Cm-s*, 50 mg/l	Liquid culture medium	100	*m*-Hydroxybenzoic acid, *m*-benzyl alcohol	7 d	Martins et al., 2003

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nature. Biodegradation of Azure B and Tropaeolin O in both nitrogen-limited and nitrogen-sufficient cultures of *P. chrysosporium* results in the formation of colored metabolites (Cripps et al., 1990). A metabolite of Congo Red was found only in nitrogen-sufficient cultures.

Sulfophthalimides (3- and 4-SPIs) were identified as the major metabolites during decolorization of the sulfophthalocyanine (SPC) textile dyes Reactive Blue 15 (RB 15) and Reactive Blue 38 (RB 38) by *Bjerkandera adusta* (Hein-fling-Weidtmann et al., 2001). HPLC with diode-array detection and HPLC-MS proved that SPIs are produced from both dyes in fungal cultures and by action with its purified MnP and LiP. The isomeric ratio of 3- and 4-SPI was about 3 : 1 during RB 15 decolorization. *Cunninghamella elegans* transformed Malachite Green up to 54 μm with a first-order rate constant of 0.029 μM/h per milligram of cells (Cha et al., 2001). Loss of color indicated that Malachite Green is reduced enzymatically to its Leucomalachite Green (LMG). Malachite Green is also converted to N-demethylated and N-oxidized metabolites, including primary and secondary arylamines. The peaks of metabolites correspond to mono-, di-, and tridesmethyl derivatives of Malachite Green and mono-, di-, tri-, and tetradesmethyl derivatives of LMG. The metabolites extracted from the culture supernatants and from mycelium-extracted samples were similar, except for Malachite Green *N*-oxide, which was detected only in the mycelia. Cytochrome P450 inhibitors suggest that the cytochrome P450 system mediates the N-demethylation and reduction reactions. This study showed that *C. elegans* has multiple pathways to transform triphenylmethane dyes by an intracellular cytochrome P450 system. The mechanism proposed for the metabolism of Malachite Green and Leucomalachite Green is shown in Figure 10.1.

Decolorization of Rose Bengal is due to adsorption to mycelium and action by LiP of *P. chrysosporium* (Gogna et al., 1992). One unidentified metabolite was detected in 0 to 5 hours. Similarly, one unknown metabolite was detected in the supernatants and mycelia of four ligninolytic basidiomycete fungi (*P. chrysosporium*, *Phellinus gilvus*, *Pleurotus sajor-caju*, and *Pycnosporus sanguineus*) due to Indigo dye degradation as the source of carbon (Balan and Monteiro, 2001). *Trametes versicolor* decolorized all different substituted bioaccessible reactive azo dyes efficiently after 7 days (Martins et al., 2003). Laccase, lower LiP activity, and glyoxal oxidase (GLOX) residual activity were detected. Decolorization of *Cm-s* resulted in the formation of two hydroxylated metabolites, *meta*-hydroxybenzoic acid and *meta*-benzyl alcohol. A metabolic pathway has been proposed based on the hydroxylated metabolites.

10.10   FACTORS AFFECTING FUNGAL DECOLORIZATION AND DEGRADATION OF DYES

The characteristics of dye wastewater are very important for the process of decolorization. A fungus may decolorize one dye and has a different capacity for other dyes. Factors such as media composition, pH, carbon and nitrogen
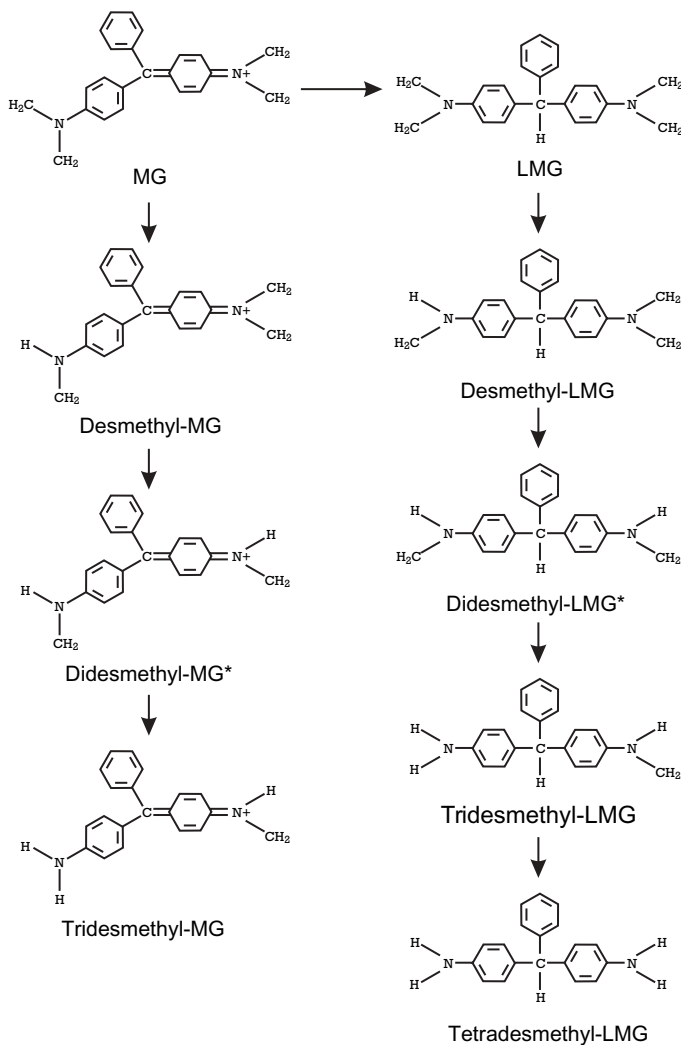
Figure 10.1 Tentative mechanism for metabolism of Malachite Green (MG) and Leucomalachite Green (LMG) by *Cunninghamella elegans*. [Reprinted from Cha et al. (2001), *Applied and Environmental Microbiology*.]

sources, TOC/N ratio, incubation time, ionic strength, and initial dyestuff concentrations have a profound effect on the rate of color removal and the process of biodegradation (Table 10.6). These factors are described below.

10.10.1 Media Composition

Of four different media, Kirk's basal salts and urea-containing media support complete decolorization of Everzol Turquoise G by *Coriolus versicolor*

TABLE 10.6 Factors Affecting Fungal Decolorization and Degradation of Dyes

Fungus	Dye Conc.	Factors	Medium	Color Removal (%)	Incubation (hours/days)	Reference
Cyathus bulleri	Crystal Violet, 54 µM		Kirk's	69	4d	Vasdev et al., 1995
			Glucose–asparagine	96		
			Maltose–mineral	96		
			Brodie's	91		
Coriolus versicolor	Everzol Turquoise Blue, 200 mg/l		Kirk's basal salts	100	6d	Kapdan et al., 2000
			Kirk's, excluding VA and Tween 80	80		
			Urea-containing medium, no VA and Tween 80	100		
			Urea-containing medium, little glucose	82		
		5 g/l Glucose	Urea-containing medium, no VA and Tween 80	100	9d	
		5 g/l Fructose		92	9d	
		Ammonium sulfate		83	6d	
		Ammonium chloride		96	6d	
		Ammonium tartrate		96	6d	
		Urea		98	6d	
		TOC/N > 20		>98	6d	

Organism	Dye	Culture condition	Medium	%	Time	Reference
Bjerkandera sp. strain BOS55	Amaranth, 50 ppm	Static/agitated	Kirk's	8/98	20/1 d	Swamy and Ramsay, 1999a
	Remazol Black B, 60 ppm			9/96	20/3 d	
Phanerochaete chrysosporium	Amaranth, 50 ppm			7/100	20/12 d	
	Remazol Black B, 60 ppm			24/100	20/15 d	
	Tropaeolin O, 20 ppm			15/89	20/13 d	
Trametes versicolor	Amaranth, 50 ppm			5/100	20/1 d	
	Remazol Black B, 60 ppm			13/100	20/2 d	
	Reactive Blue, 20 ppm			0/100	7 d	
	Remazol Orange, 60 ppm			0/100	3 d	
	Remazol Brilliant Blue, 40 ppm			0/100	1 d	
Bjerkandera fumosa	Basic Blue 22, 0.1 g/l	Static/agitated	Glucose-yeast extract	20/100	14 d/2 h	Jarosz-Wilkolazka et al., 2002
	Acid Red 183, 0.1 g/l			8/68	14 d/7 d	
Kuehneromyces mutabilis	Basic Blue 22, 0.1 g/l			18/100	14 d/1 d	
	Acid Red 183, 0.1 g/l			6/100	14 d/7 d	
Stropharia rugosoannulata	Basic Blue 22, 0.1 g/l			14/100	14 d/14 h	
	Acid Red 183, 0.1 g/l			0/100	7 d	
Irpex lacteus	Remazol Brilliant Blue R, 150 µg/ml	Stationary Agitated submerged	Mineral medium	100	10 d	Kasinath et al., 2003
				95		
I. lacteus	Remazol Brilliant Blue R, 200 mg/l	Stationary	Low-nitrogen mineral medium	95	14 d	Novotny et al., 2004
	Reactive Orange 16, 200 mg/l			96		

TABLE 10.6 *Continued*

Fungus	Dye Conc.	Factors	Medium	Color Removal (%)	Incubation (hours/days)	Reference
	Reactive Black 5, 200mg/l			98		
	Naphthol Blue Black, 200mg/l			99		
	Chicago Sky Blue, 200mg/l			98		
	Disperse Blue 3, 200mg/l			94		
	Cu-Phthalocyanine, 200mg/l			97		
	Bromophenol Blue, 200mg/l			96		
Aspergillus foetidus	Drimarene Red, 50mg/l	1% Glucose	Mineral salts	90	40h	Sumathi and Manju, 2000
	Drimarene Navy Blue, 50mg/l			>99	60h	
	Drimarene Red, 50mg/l	0.5% Starch	Mineral salts	98	72h	
	Drimarene Navy Blue, 50mg/l			98	72h	
	Drimarene Red, 50mg/l	5mg/l Cr(VI) and 1% NaCl	Mineral salts	>97	72h	
Funalia trogii (pellets, first cycle)	Astrazone Blue, 264mg/l	4g/l Glucose 20% Cheese whey	Distilled water	94 93	24h	Yesilada et al., 2003
Debaryomyces polymorphus	Reactive Black 5, 200mg/l	5g/l Glucose and 0.5–1.0g/l $(NH_4)_3 PO_4$	Liquid medium	>99	24h	Yang et al., 2005

MUCL (Kapdan et al., 2000). *Trametes versicolor* decolorized Direct Red 16 and Acid Blue 113 completely on a malt extract agar plate containing 10 ppm each (Hardin et al., 2000). *Pleurotus ostreatus* and *P. chrysosporium* decolorized these dyes in 5 to 6 days on Kirk's medium.

10.10.2 Static Versus Agitated Culture Conditions

Oxidative enzymes of white-rot fungi are responsible for biodegradation, which functions optimally in the presence of 100% oxygen (Swamy and Ramsay, 1999a). In static cultures, a surface mat limited oxygen transfer to the cells beneath the surface and in the medium. An agitated approach is practical for the treatment of a large volume of wastewater. *Geotrichum* sp. CCMI 1019 revealed the most favorable decolorization rates of reactive azo dyes at 28ºC and agitation rates of 120 rpm (Maximo et al., 2003). *Irpex lacteus* showed higher levels of LiP, MnP, and laccase in stationary than in submerged cultures (Kasinath et al., 2003). MnP plays a major role in the decolorization of Remazol Brilliant Blue R. MnP and laccase of *I. lacteus* appear to be involved in the decolorization of anthraquinone and azo dyes in shallow stationary cultures (Novotny et al., 2004).

10.10.3 pH and Temperature

Trametes versicolor and isolate DSPM95 maintained the degradation of Poly R-478 over a wide range of pH, up to pH 6.0 (Tekere et al., 2001). Decolorization was not affected by pH (Chagas and Durrant, 2001). The pH range 3 to 10 was shown to have no correlation with dye adsorption (Mou et al., 1991; Brahimi-Horn et al., 1992). Buffers and pH influenced the decolorization of several dyes by *T. versicolor* (Swamy and Ramsay, 1999a). Decolorization of all dyes, as the first or second dye, occurred due to 2,2′-dimethyl succinate (2,2′-DMS) buffer. The ineffectiveness of buffers such as citrate–phosphate and sodium tartrate may be due in part to their consumption as carbon sources. The addition of dye caused the loss of buffering capacity by all buffers except 2,2′-DMS. A pH value of 5 reduced the efficiency to 80% and a pH between 6 and 7 reduced it to 50% (Kapdan et al., 2000). Various textile and dye effluents are produced at relatively high temperatures, between 50 and 60ºC, and thus temperature is an important factor in dye decolorization. An optimum temperature for the growth of isolates ranges from 25 to 37ºC (Tekere et al., 2001). *T. versicolor* CNPR 8107 decolorized Remazol Blue RR at a higher rate at 30ºC than at 37ºC 2 days after dye addition (Toh et al., 2003).

10.10.4 C and N Sources, TOC/N Ratios, and Salts

Biodegradation of the dyes can be enhanced by improving the initial cultural conditions. In one study, the optimum concentration of glucose was 15 g/l for

decolorization of nitrated stilbene–sulfonic acid effluent by *Coriolus versi-color* (Knapp and Newby, 1999). Color removal of Congo Red was suppressed with a high dose of nutrient nitrogen (Tatarko and Bumpus, 1998). Nitrogen had no effect on the decolorization of dyes by *Cyathus bulleri* (Vasdev et al., 1995). More effective decolorization also occured in the presence of yeast extract (Zhou and Zimmermann, 1993).

A glucose concentration of 0.3% produced a strong effect on decoloriza-tion, and the organic nitrogen in the effluent was adequate for the process by *Aspergillus niger* (Assadi and Jahangiri, 2001). An inoculum size of up to 10% rapidly enhanced the rate of decolorization. Urea performed better (98%) color removal than did ammonium salts for the decolorization of Everzol Turquoise Blue G by *Coriolus versicolor* MUCL (Kapdan et al., 2000). Almost complete color removal occurred at TOC/N ratios between 112 and 22, or glucose concentrations between 5000 and 1000 mg/l after 6 days. Glucose, starch, maltose, and cellobiose were shown to be good carbon sources for decolorization of the cotton bleaching effluent by an unidentifed white-rot fungus (Zhang et al., 1999). However, the decolorization rate was lowered by the addition of NH_4^+. Sucrose or both sucrose and peptone sig-nificantly enhanced the decolorization of the reactive azo dyes by *Cunning-hamella elegans* UCP 542 (Ambrosio and Campos-Takaki, 2004). However, the presence of only nitrogen in the solutions suppressed the decolorization. *P. chrysosporium* caused maximum decolorization of Methyl Violet at a glucose concentration of 5 g/l, 0.05 g/l of ammonium chloride, an inoculum size of 3.2×10^5 cells/ml, pH 4 to 5, at 35ºC (Radha et al., 2005). Peptone supported better decolorization of Remazol Brilliant Violet by *Coriolus ver-sicolor* (Sanghi et al., 2006).

10.10.5   Initial Dye Concentration

It is important to optimize an initial dye concentration for color removal. This aspect was discussed in portions of the foregoing text. Higher dye concentra-tions are always toxic. The decolorization efficiency was shown to be 100 and 80% at a dyestuff concentration between 100–500 and 700–1200 mg/l, respec-tively, by *C. versicolor* (Kapdan et al., 2000). Only 12% decolorization occurred at a dye concentration of 1650 mg/l. *Pseudozyma rugulosa* Y-48 and *Candida krusei* G-1 decolorized 99% of 200 mg/l Reactive Brilliant Red K-2BP, respectively (Yu and Wen, 2005). Dye decolorization was reduced to 20% at a concentration of 1000 mg/l by these yeast strains.

10.11   FUNGAL DYE DECOLORIZATION AND DEGRADATION BIOREACTORS

Only in recent years have fungal decolorization and degradation bioreactors of dyes been developed (Table 10.7). These processes require scaling in the

TABLE 10.7 Fungal Bioreactors for Decolorization and Degradation of Dyes

Fungus	Bioreactor Configuration	Bioreactor Volume	Dye Conc.	Color Removal (%)	Enzyme Activity (U/ml)	Duration (minutes/hours/days)	Reference
Phanerochaete chrysosporium	Rotating drum (nylon sponge cubes)		Poly R-478	19	MnP 1350, LiP 364	15 m	Dominguez et al., 2001
Trametes versicolor (encapsulated)	Stirred-tank reactor	1 L nonsterile	Poly R-478, 200 mg/l	80		41 d	Leidig et al., 1999
T. versicolor (free pellets)	Stirred-tank reactor	4 L	Reactive Black 5 Reactive Red 198 Reactive Blue 19, 100–500 mg/l	>97 >97 >91		200 d	Borchert and Libra, 2001
	Agitated sequencing batch	Nonsterile 500-ml flask	Reactive Black 5, 100 mg/l	>97		200 d	
P. chrysosporium	Column	31 cm × 5 cm	Crystal Violet (0.002%)	92		3 d	Das et al., 1995
Pycnosporus cinnabarinus	Packed-bed (nylon web cubes)	10 cm diameter, 20 cm long void vol. 1.6 L	Pigment plant effluent	clear	Laccase	3 d	Schliephake et al., 1993
Fungus F29	Continuous packed bed	25 cm height 4 cm diameter, 1 L	Orange II, 1000 mg/l	95		2 m	Zhang et al., 1999
Fungus F29 (pellets)	Fed-batch fluidized bed	1 L	Orange II, 2000 mg/l	>95		1 m	
Immobilized fungus F29	Fed-batch fluidized bed	1 L	Orange II, 2000 mg/l	97		24 h	

TABLE 10.7 *Continued*

Fungus	Bioreactor Configuration	Bioreactor Volume	Dye Conc.	Color Removal (%)	Enzyme Activity (U/ml)	Duration (minutes/ hours/days)	Reference
Free fungus F29 (pellets)	Continuous fluidized bed	1 L	Orange II, 2000 mg/l	95–97		17 d	Blanquez et al., 2004
T. versicolor (pellets)	Fluidized, batch continuous	1.5 L	Grey Lanaset G, 0.15 g	90		5 d	Kapdan and Kargi, 2002a
Coriolus versicolor	Immobilized on RBC, repeated batch	1.7 L	Everzol Turquoise Blue G, 50–200 mg/l	90 80		40 d 12 d	Ge et al., 2004
Phanerochaete sordida	Immobilized on RBC, repeated batch	1.61 L	Basic Blue 22, 200 mg/l	98		24 h	Kapdan and Kargi, 2002b
Coriolus versicolor	Immobilized in form of biofilm, activated sludge, and wood ash	Aeration tank 8.3 L and sedimentation tank, 1.7 L	Everzol Turquoise Blue G, 200 mg/l	82		20 d	Mielgo et al., 2001
P. chrysosporium	Immobilized on polyurethane foam, pulsed packed bed	167 ml, scaling to 2 L	Orange II	>99	MnP, 14 U/l	24 h	Mielgo et al., 2002
P. chrysosporium	Immobilized on polyurethane foam, pulsed packed bed	167 ml	Poly R-478, 0.1 g/l	80	MnP 126 U/l	24 h	

Organism	Treatment	Volume	Dye	%	Enzymes	Time	Reference
P. chrysosporium	Immobilized on polyurethane foam, pulsed packed bed	167 ml	Poly R-478, 100 mg/l	73	MnP, 100 U/l per day	20–35 d	Palma et al., 1999
P. chrysosporium	Immobilized agitated culture	10 L, 250-ml flask	Dispersed Red 533 250 ppm 500 ppm 1000 ppm	ca. 95 → 90 ca. 95 → 30 ca. 80 → 10		16 d	Yang and Yu, 1996a
P. chrysosporium	Immobilized on foam, fixed-film reactor	10 L	Dispersed Red 533, 250 ppm	88		2 d	Yang and Yu, 1996b
Chrysosporium lignorum	Immobilized in airlift fermentor	2 L	Poly R-478, 50 mg/l Poly S-119, 50 mg/l	80 95	MnP, LiP	12 d	Buckley and Dobson, 1998
Irpex lacteus	Immobilized on PUF or PW cubes, packed-bed reactor	Glass, 27 ml	Remazol Brilliant Blue R, 150 mg/l	97 100	MnP and laccase	10 d 6 d	Kasinath et al., 2003
I. lacteus	Immobilized on PW cubes, packed-bed reactor	Glass, 27 ml	Remazol Brilliant Blue R, 150 mg/l	100	MnP and laccase	6 d	Novotny et al., 2004
P. chrysosporium	Immobilized in ZrOCl$_2$-activated pumice, packed-bed reactor	Glass column 1.5 cm in diameter and 10 cm in height	Direct Blue, 20 mg/l	100	MnP	3 d	Pazarlioglu et al., 2005

light of different optimum operating conditions. The process is basically the removal of dye color in effluents accompanied by degradation. Both processes can take place individually or simultaneously. White-rot fungi are used primarily in decolorization bioreactor systems, but their commercial applications in large-scale decolorization treatments are not known at the present time. This is because of the lack of a bioreactor system providing a constant production of high amounts of ligninolytic enzymes under continuous optimum conditions and controlled growth of fungi. Little is known about decolorization bioreactor systems that use yeasts. Fungal decolorization and degradation bioreactors of dyes can be classified into the types discussed below.

10.11.1   Rotating Drum, Stirred-Tank, and Membrane Bioreactors

A new bioreactor configuration based on the standard rotating drum bioreactor has been developed for the production of ligninolytic enzymes in semisolid conditions (Dominguez et al., 2001). This configuration is suitable for the continuous production of high titers of LiP for a long time without operational problems. About 19% Poly R-478 was decolorized after 15 minutes by an extracellular fluid of the bioreactor, containing mainly MnP of *Phanerochaete chrysosporium*. *Trametes versicolor* decolorized three reactive dyes in a sequencing batch process in the presence of glucose over an extended period without supplementation by a new mycelium (Borchert and Libra, 2001). Decolorization is attributed to the production of peroxidases and can be reactivated continuously by sheering the suspended pellets. A mycelium of *Coriolus versicolor* removed color (95%) and COD (75%) in a batch reactor, and fungal pellets could be used for eight cycles (Sanghi et al., 2006). Crystal Violet decolorized in a column bioreactor using *Phanerochaete chrysosporium* (Das et al., 1995). The peak decolorization was reached in 3 days with glucose compared to 4 to 6 days with sucrose. A membrane bioreactor using *T. versicolor* combined with reverse osmosis was effective for decolorization and organic removal of dye wastewater (Kim et al., 2004).

10.11.2   Packed- and Fluidized-Bed Bioreactors

A wood-rotting fungus strain F29, an unidentified basidiomycete, decolorized Orange II under agitated conditions (Knapp et al., 1997). The fungus showed retention of high degradative activities along with reuse of the mycelia and thus indicated its possible value in continuous bioreactor systems. Fungal strain F29 decolorized 95 to 99% Orange II in a continuous packed-bed and fed-batch fluidized-bed bioreactor systems (Zhang et al., 1999). The fed-batch bioreactor consisted of a conical vessel (1-L working volume) with a lid and three tubes, each for media, sampling, and air outlet, respectively, and is shown in Figure 10.2. Orange II also decolorized in this bioreactor with the use of immobilized cells for 27 cycles for 2 months. No relationship existed
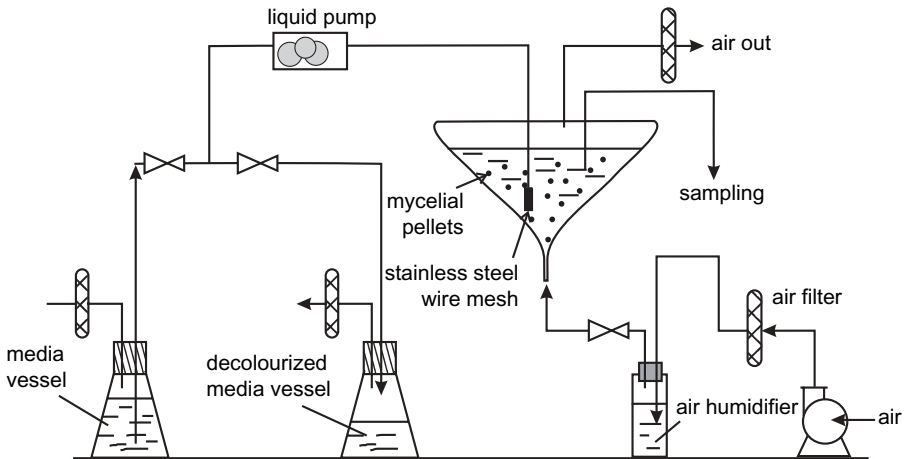
Figure 10.2 Fedbatch fluidized-bed reactor using fungal strain F29 mycelial pellets for decolorization of dyes. [Reprinted from Zhang et al. (1999), copyright © with permission from Elsevier.]

between the laccase activity of *T. versicolor* pellets and the rate of decolorization of a metal-complexed dye, Grey Lanaset G, in a continuous fluidized-bed bioreactor (Blanquez et al., 2004). The pellets became dark due to the dye adsorbed on the biomass. Degradation appeared to be related to intracellular enzyme activity.

10.11.3 Immobilized Bioreactors

In general, immobilized systems exhibit good biological activities and capabilities for long-term operation. Decolorization of Poly R-478 and Poly S-119 has been attributed to LiP and MnP activities by an immobilized *Chrysosporium lignorum* CL1 in a 2-L airlift bioreactor or cultivated under static conditions (Buckley and Dobson, 1998). A MnP activity of *P. chrysosporium* was shown to be responsible for effective Orange II and Poly R-478 decolorization (Mielgo et al., 2001, 2002). Decolorization not only depends on the MnP activity of *P. chrysosporium* but also on cofactors influencing the catalytic activity of this enzyme (Palma et al., 1999). Another cofactor, hydrogen peroxide, plays a major role in decolorization efficiency. Additional research on the discovery of new cofactors and their role in mechanism of decolorization evolves into new applications in the biotechnological processes by fungi. Figure 10.3 shows a fixed-film bioreactor system for continuous decolorization of Red 533 Dispersed dye using immobilized *P. chrysosporium* cells (Yang and Yu, 1996b). The process can operate continuously for 10 to 20 days or more with a decolorization efficiency exceeding 88% at an initial dye
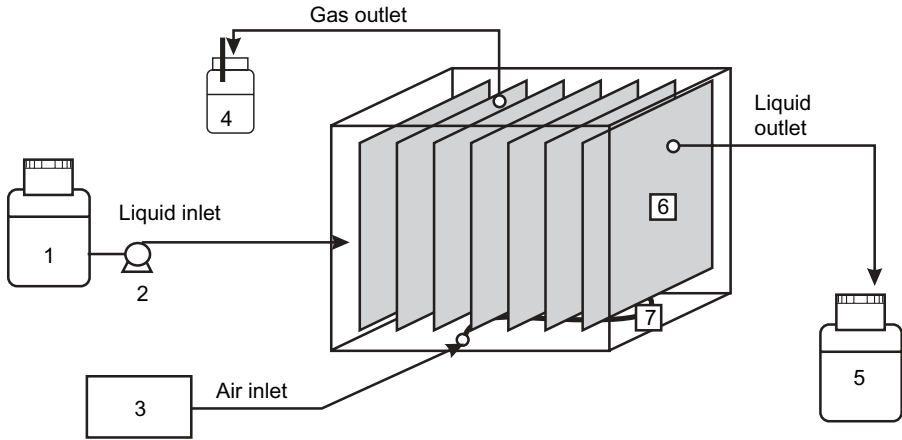
Figure 10.3 Fixed-film bioreactor system for continuous decolorization of Red 533 Dispersed dye using immobilized *Phanerochaete chrysosporium* cells. 1, Feeding media reservoir; 2, peristaltic pump; 3, air compressor; 4, offgas condenser; 5, effluent collector; 6, fixed films (foams); 7, air sparger. [Reprinted from Yang and Yu (1996b), copyright © with permission from Springer Science and Business Media and the authors.]

concentration of 250 ppm and 2 days of retention time. *Irpex lacteus* immobilized in a pine wood (PW) reactor decolorized Remazol Brilliant Blue R more rapidly (100% in 6 days) than did a polyurethane foam (PUF) reactor (Kasinath et al., 2003). MnP was produced five times more in a PUF than in a PW reactor. Laccase activities were similar in both reactors. Both reactors also decolorized four different color bath effluents. MnP and laccase activities were present during decolorization of Remazol Brilliant Blue R by *Irpex lacteus* immobilized in pine wood (Novotny et al., 2004). Immobilized *Funalia trogii* on *Luffa cylindrica* sponge decolorized Reactive Black 5 (RB 5) completely at initial dye concentrations (15, 18, 34, and 61 mg/l) added to a 30-day-old culture medium (Mazmanci and Unyayar, 2005). However, complete decolorization occurred within 48 hours in media containing 96 and 125 mg/l. No carbon or nitrogen source in the media was necessary when *Luffa cylindrica* was used for immobilization.

10.12   DECOLORIZATION AND DEGRADATION OF DYES BY FUNGAL ENZYMES

Present knowledge on decolorization of dyes by fungal enzymes is based on enzymes secreted by white-rot fungi. The extracellular enzymes include two peroxidases, LiP and MnP, and laccase. These enzymes can cleave the aromatic rings and have the potential to remove color from the dyes. Commercial products of laccase are known, but LiP and MnP are not commercially avail-

able at the present time. One drawback is that they can attack one type of dye molecules and may block attacking another dye structure.

10.12.1 Peroxidase-Catalyzed Decolorization and Degradation of Dyes

Lignin peroxidases are important enzymes of the lignin-degrading system and are involved in the degradation of dyes (Table 10.8). Azure B, Tropaeolin O, and Orange II were partially decolorized within 20 minutes by crude LiP during nitrogen-limited cultures of *P. chrysosporium* (Cripps et al., 1990). Isolated isozymes designated as H1, H2, H8, and H10 of LiP showed similar results of decolorization. Decolorization of dyes with crude LiP resulted in the formation of a number of metabolites. A number of differences exist between metabolites formed during decolorization of dyes by fungal cultures or with LiP. This suggests the expression of many enzymes or enzyme systems other than LiP during initial steps or biodegradation of dyes. The efficiency of enzymatic decolorization was lower than that of the whole culture during mineralization of five azo dyes and sulfanilic acid by *P. chrysosporium* (Paszczynski et al., 1992). LiP H8 of *P. chrysosporium* cultures displayed the formation of a number of oxidation products upon incubation of Congo Red at pH 3.0 (Tatarko and Bumpus, 1998). The decolorization of dyes increased with higher LiP concentrations (Wu et al., 1996). The addition of VA enhanced the decolorization of Reactive Red 22 to 96% within 3 minutes. Isozymes of LiP from *P. chrysosporium* decolorized dyes in the presence of VA similar to crude LiP (Ollikka et al., 1993). LiP from *Trametes versicolor* decolorized Remazol Brilliant Blue R (RBBR) in the presence or absence of VA (Christian et al., 2005). However, VA stabilized and improved the rate of RBBR decolorization. A mechanism has been described for the role of VA in the decolorization of RBBR by LiP.

LiP from *P. chrysosporium* catalyzed the degradation of Methylene Blue and Azure B via N-demethylation oxidation (Ferreira et al., 2000). LiP oxidation of Methylene Blue produced nearly 70% of the mono- and didemethylated derivatives Azure B and Azure A. A 1:10 dye:H_2O_2 relation showed nearly 80% decolorization, suggesting its application in wastewater treatment. It has been shown that a crude exoenzyme preparations from *Trametes versicolor* and *Bjerkandera adusta* can decolorize Reactive Blue 38 and Reactive Violet 5 (Heinfling et al., 1997). MnP isozymes from *B. adusta* and *Pleurotus eryngii* decolorized azo dyes and phthalocyanine complexes in a Mn(II)-independent reaction (Heinfling et al., 1998). Dye oxidations were enhanced by the addition of VA as in LiP reactions. Decolorization of Reactive Blue 15 accounted for 60% of sulfophthalimides by purified MnP from *B. adusta* (Heinfling-Weidtmann et al., 2001). A scheme has been proposed for the oxidative fission of Reactive Blue 15 by peroxidases of *B. adusta*.

LiP from *P. chrysosporium* oxidizes sulfonated azo dyes, generating sulfophenyl hydroperoxides (Chivukula et al., 1995). A proposed mechanism of

TABLE 10.8 Peroxidase-Catalyzed Decolorization and Degradation of Dyes

Fungus	Peroxidase Conc.	Dye Conc.	Medium/ Bioreactor Configuration	Color Removal Rate (%)	Metabolic Products	Incubation (minutes/ hours)	Reference
Phanerochaete chrysosporium	Crude LiP	Azure B		84	Several unknown	20 m	Cripps et al., 1990
		Tropaeolin O		46			
		Orange II		18			
P. chrysosporium	Crude LiP, 0.00673 U/ml	Reactive Red 22, 30 mg/l	0.45 mM H_2O_2	15	One unknown	5 m	Wu et al., 1996
		Evans Blue, 30 mg/l		13		5 m	
		Reactive Red 22, 30 mg/l	0.45 mM H_2O_2 and 1.28 mM VA	96		3 m	
P. chrysosporium	0.1 U LiP	Bromophenol Blue, 10–80 µM	1 ml vol.	93		15 m	Ollikka et al., 1993
		Congo Red, 10–80 µM	0.4 mM H_2O_2 and 2 mM VA	54			
		Methylene Blue, 10–80 µM		84			
		Methyl Green, 10–80 µM		86			
		Methyl Orange, 10–80 µM		85			
		Remazol Brilliant Blue R, 10–80 µM		77			
		Toluidine Blue, 10–80 µM		79			
		Poly R-478, 0.002%		46			
		Poly S-119, 0.002%		76			
		Poly T-128, 0.002%		48			
P. chrysosporium	0.125 mg LiP	Disperse Yellow 3, 0.8 µM	2.5 ml vol., 0.8 µM H_2O_2		4-Methyl-1,2-benzoquinone, acetanilide, a dimer	2 h	Spadaro and Renganathan, 1994

Organism	Enzyme	Dye (conc.)	%	Conditions	Products	Time	Reference
P. chrysosporium	1.2 µM LiP	Azo dye I, 1 µM		1 ml vol. 1 µM H_2O_2	2,6-Dimethyl-1,4-benzoquinone, 4-sulfophenyl hydroperoxide	1 h	Chivukula et al., 1995
		Orange II, 1 µM (azo dye II),			1,2-Naphthoquinone, 4-sulfophenyl hydroperoxide		
		Azo dye III, 1 µM			1,2-Naphthoquinone, 2,6-dimethyl-4-sulfophenyl hydroperoxide		
		Azo dye IV, 1 µM			2,6-Dimethyl-1,4-benzoquinone, 4-sulfonamidophenyl hydroperoxide		
White-rot fungus	0.5 U/ml LiP	Indigo Carmine, 50 mg/l	98	0.6 mM H_2O_2, 1 mM VA		1 h	Young and Yu, 1997
		Reactive Blue, 50 mg/l	94				
		Acid Violet, 50 mg/l	93				
		Reactive Black 5, 50 mg/l	91				
		Acid Green 27, 50 mg/l	85				
		Acid Blue 25, 50 mg/l	82				
		Acid Orange 74, 50 mg/l	79				
		Acid Black 24, 50 mg/l	25				
Pleurotus ostreatus	0.01 U LiP	Congo Red, 10–40 µM	32	3 ml vol., 0.1 mM H_2O_2		5 m	Shin and Kim, 1998
		Methyl Orange, 10–40 µM	96				
		Poly R-478, 10–40 µM	28				

TABLE 10.8 *Continued*

Fungus	Peroxidase Conc.	Dye Conc.	Medium/ Bioreactor Configuration	Color Removal Rate (%)	Metabolic Products	Incubation (minutes/ hours)	Reference
		Crystal Violet, 10–40 μM		74			
		Malachite Green, 10–40 μM		46			
		Bromophenol Blue, 10–40 μM		98			
		Methylene Blue, 10–40 μM		10			
		Toluidine Blue O, 10–40 μM		10			
P. chrysosporium	140 U/ml	Methylene Blue, 33 μM	1:1 MB:H_2O and 1:2 MB:H_2O Higher MB:H_2O 1:10 MB:H_2O	80	Azure A, Azure B Several products	40 m	Ferreira et al., 2000
		Azure B, 42 μM	1:1 MB:H_2O 1:2 MB:H_2O 1:10 AB:H_2O	80	Azure A Several products		
Bjerkandera adusta	200 ng/ml MnP	Reactive Blue 15 16 μM/l	0.6 mM H_2O_2		Sulfophthalimides	30 m	Heinfling-Weidtmann et al., 2001
Bjerkandera sp.	150–200 U/l MnP	Orange II	H_2O_2	80		40 m	Lopez et al., 2002
P. chrysosporium	MnP, 1000 U/l	Poly R-478, 0.03 g/l	Fixed-bed tubular with cubes of nylon sponge	24		15 m	Moldes et al., 2003
	MnP 1 U, 10 μl H_2O_2	Poly R-478, 0.12 g/l Crystal Violet, 0.07 g/l	sodium malonate, MnSO₄, 1 ml vol. 20 ml vol.	30 70		2 h	

LiP-catalyzed oxidation of sulfonated azo dye is shown in Figure 10.4. Two successive one-electron oxidations of the phenolic ring of azo dyes I, II, III, or IV by H_2O_2-oxidized forms of LiP produce a carbonium ion. Water attacks the phenolic carbon of the azo linkage, forming an unstable hydroxy intermediate that breaks down to produce a quinone and sulfo- or sulfonamidophenyldiazene. Phenyldiazene oxidizes by oxygen to produce a phenyldiazene radical that removes nitrogen to yield a sulfo- or sulfonamidophenyl radical. These radicals are scavenged by oxygen to form the corresponding phenyl hydroperoxides. *P. chrysosporium* crude LiP oxidation of the sulfonated azo dye 4-(4′-sulfophenylazo)-2,6-dimethylphenol identifies 2,6-dimethyl-1,4-benzoquinone, 4-nitrosobenzenesulfonic acid, 4-aminobenzenesulfonic acid, 4-hydroxybenzenesulfonic acid, and benzenesulfonic acid (Goszczynski et al., 1994). Oxidation of 4-(4′-sulfonamidophenylazo)-2-methoxyphenol yields 2-methoxybenzoquinone, benzenesulfonamide, 4-aminobenzenesulfonamide, 4-hydroxybenzenesulfonamide, and 2-methoxy-4-aminophenol.

The rates of decolorization of all eight dyes was shown to increase linearly with the LiP doses, but the slopes depended on the dye structures (Young and Yu, 1997). Partially purified LiP along with VA, H_2O_2, and acidic pH (3.5 to 5) exhibited high rates of decolorization. The eight dyes can be classified into two groups based on the effect of H_2O_2 on dye decolorization: One has an optimum H_2O_2 concentration and decolorization declines with an overdose, and the other has an enhanced decolorization with high H_2O_2 concentration. The nature of the substituents in dye affected the enzymatic activity in fungi, and the hydroxyl and amino groups enhanced decolorization (Abadulla et al., 2000a). The presence of LiP and/or MnP in addition to laccase produced by *Neurospora crassa*, *Pleurotus ostreatus*, *Schizophyllum commune*, and *Sclerotium rolfsii* enhanced up to 25% decolorization of all azo dyes, Acid Blue 72 (Indigo), Reactive Blue 10 (Anthraquinone), and Basic Red 9 Base (Triarylmethane). Various salts, such as sodium acetate and sodium chloride, reduced the decolorization and partially precipitated the proteins. Dyeing auxiliaries, such as Cibacel DBC, lowered the decolorization efficiency.

Seven fungal strains decolorized three structurally diverse dyes (Moreira et al., 2000). All seven fungi secrete MnP, and laccase activity was detected only in *Phlebia radiata*. *P. ostreatus* produced an extracellular peroxidase that can decolorize Remazol Brilliant Blue R (Shin et al., 1997). This peroxidase decolorized eight different dyes, including triphenylmethane, heterocyclic, azo, and polymeric dyes, to a certain extent after 5 minutes (Shin and Kim, 1998). Some dyes were decolorized with a crude preparation of LiP from *P. chrysosporium* so fast that it was difficult to follow the kinetics of the enzymatic reaction (Podgornik et al., 1995). LiPs of *P. chrysosporium* decolorized dyes of different structures and classes, regardless of their charge (Podgornik et al., 1999). In general, the maximum rate of decolorization was proportional to LiP activity, whereas the time of disappearance depended on both LiP activity and dye concentration. LiP from *P. chrysosporium* decolorized about
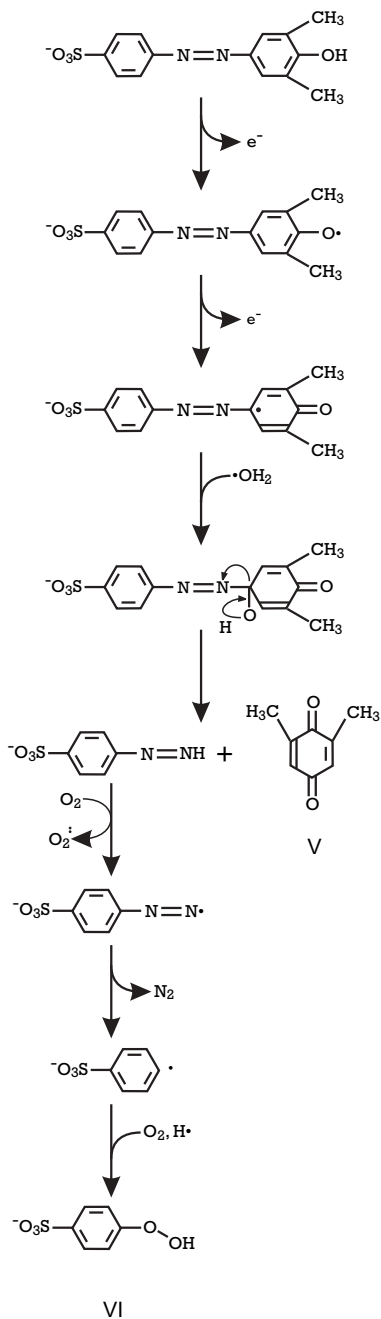
Figure 10.4 Lignin peroxidase–catalyzed oxidation of sulfonated azo dye. [Reprinted from Chivukula et al. (1995), copyright © with permission from the American Chemical Society.]

30% Remazol Brilliant Blue R in a 1-ml reaction volume, but scaling up the reaction was not linear (Peralta-Zamora et al., 1999). Sulfonphthalein (SP) dyes were decolorized by MnP and manganese-independent peroxidase (MIP) of *Pleurotus ostreatus* (Shrivastava et al., 2005). The MnP-catalyzed decolorizing activity for SP dyes is in the preferred order Phenol Red > *ortho*-Cresol Red > *meta*-Cresol Purple > Bromophenol Red > Bromocresol Purple > Bromophenol Blue > Bromophenol Green. MIP-catalyzed decolorizing activity for SP dyes is in the order Bromocresol Green > Bromophenol Blue > Bromocresol Purple > *meta*-Cresol Purple > *ortho*-Cresol Red > Phenol Red.

Little is known of the influence of dye concentration on the rate of color removal by peroxidases, but a general indication is that high dye concentration is responsible for the slow rate of color removal. In general, the decolorization rate in all eight dyes was inhibited at high dye concentrations (Young and Yu, 1997). High dye concentration require more attacks by LiP radicals, due to a slower rate of color removal. Based on the molecular ratio of H_2O_2 to dye, about 36 attacks of LiP compounds I and II will be required for the disappearance of color of Reactive Black 5. This implies that a plug-flow bioreactor containing biofilters may not be efficient in dye color removal, due to high inlet concentrations. Thus, a bioreactor with a controlled back-mixing flow of wastewater is to be designed to cover the effects of substrate inhibition and H_2O_2 overdose.

Poly R-478 has been decolorized in vivo by semisolid cultures of *P. chrysosporium* using corncobs (Rodriguez Couto et al., 2000). About 85% Poly R-478 (0.02 w/v) was decolorized using VA, and a lower percentage of 58% occurred in MnO_2 cultures. A correlation existed between decolorization of Poly R-478 and MnP activity. Little activity of MnP was involved in dye decolorization, suggesting that Mn(III) is a less efficient oxidative agent than LiP cation radicals (compounds I and II) (Young and Yu, 1997).

Anthraquinone dye could be decolorized in shaken flasks after 7 days by *Geotrichum candidum* Dec 1 (Kim and Shoda, 1999a). A novel peroxidase (DyP) involved in the decolorization of dyes produced by *Geotrichum candidum* Dec 1 was purified and described by Kim and Shoda (1999b). Nine of the 21 dyes, especially the anthraquinone dyes decolorized by Dec 1 cells, were also decolorized by DyP.

10.12.1.1 Peroxidase Bioreactors. The use of enzyme-based treatment of dyes in wastewaters is rather new and unusual. Enzymatic membrane reactors can be a promising technology to achieve such objectives. Orange II decolorized in a continuous stirred-tank reactor using MnP of *Bjerkandera* sp. in a polyethersulfone membrane (Lopez et al., 2002). The preliminary experiment employed the addition of fresh MnP in pulses in fed-batch operation to maintain the highest degree of decolorization. Continuous decolorization was performed by continuous addition of MnP, H_2O_2, and dye. The system decolorized 95% at a high dye loading rate of 2400 g/m^3 per day for 20 minutes and reached a steady-state higher than 90% after 40 minutes.

MnP of *P. chrysosporium* produced in fixed-bed tubular bioreactors was shown to have the ability to decolorize structurally different dyes (Moldes et al., 2003). The bioreactor consisted of a glass column (0.25-L working volume) filled with cubes of nylon sponge operated under semisolid conditions. During continuous addition of MnP and H_2O_2 in a total volume of 20 ml, Poly R-478 and Crystal Violet were decolorized 30% and 70%, respectively, after 2 hours. Photochemical treatment degraded 40% of Crystal Violet in 2 hours.

10.12.2 Laccase-Catalyzed Decolorization and Degradation of Dyes

In addition to LiP and MnP, laccase plays an important role in dye decolorization. Fungal laccase-catalyzed decolorization and degradation of various types of dyes are noted in Table 10.9. Laccase from *Pyricularia oryzae* oxidized a number of phenolic azo dyes (Chivukula and Renganathan, 1995). Reduction of these azo linkages resulted in the formation of potentially carcinogenic aromatic amines. VA acted as an inducer of laccase by *Botryosphaeria* sp. when screened on the polymeric dye Poly R-478 (Barbosa et al., 1996). *Phlebia tremellosa* decolorized more than 96% (initial concentration 200 mg/l) of textile azo dyes within 14 days under stationary incubation conditions (Kirby et al., 2000). Laccase activity was revealed in culture supernatants in both the presence and absence of textile dye and correlated well with the period of dye decolorization. The supernatants of high laccase activity of *Fomes sclerodermeus* decolorized Malachite Green in the presence or absence of 1-hydroxybenzotriazole (1-HBT) (Papinutti and Forchiassin, 2004). This treatment produced a colorless substance named Decolorized Malachite Green (DMG), which is not toxic to *P. chrysosporium* or any other white-rot fungi.

All strains of *Pleurotus ostreatus* attain high laccase and MnP activities and decolorize Reactive Blue 158, Acid Blue 185, Acid Black 194, Orisol Blue BH, and Orisol Turquoise JL to various extents (Rodriguez et al., 1999). *Trametes hispida* showed the highest laccase volumetric and decolorization activities on all five dyes. Only the laccase activity correlated with the rate of decolorization in crude extracts. Two laccase isozymes purified from *T. hispida* can also decolorize these dyes. A mixture of two purified laccases (POXC and POXA3) from *Pleurotus ostreatus* improved the decolorization rate of Remazol Brilliant Blue B and reduced the dye toxicity by 95% (Palmieri et al., 2005). Purified laccase from *Pycnosporus cinnabarinus* broke down the chromophore of Chicago Sky Blue in the presence of oxygen and produced two intermediate products (Schliephake et al., 2000). The transformation rate of dye increased with increasing concentration of laccase. Laccase from *Pycnosporus sanguineus* correlated with the dye decolorization, and less than 3% accounted for the sorption of dye to fungal mycelia (Pointing and Vrijmoed, 2000).

Differences in the decolorization efficiency of four laccases (from three species of *Trametes* and *Sclerotium rolfsii*) has been attributed to the

TABLE 10.9 Laccase-Catalyzed Decolorization and Degradation of Dyes

Fungus	Laccase Conc./Mediator	Dye Conc.	Medium/Type of Bioreactor	Color Removal (%)	Metabolic Products	Incubation (minutes/hours)	Reference
Pycnosporus cinnabarinus	0.2 mg	Chicago Sky Blue, 500 mg/l	1 ml vol.		Two unidentified products	20h	Schliephake et al., 2000
Trametes modesta	0.2 nkat/ml	Acid Blue 74	4 ml vol.	58		6h	Nyanhongo et al., 2002
		Acid Blue 225		48			
		Basic Red 9 Base		65			
		Direct Blue 71		48			
		Reactive Blue 9		98			
		Reactive Blue 221, 250mg/l		100			
	+HBT, 1mM	Acid Blue 225		100		3h	
		Acid Violet 17		100			
		Direct Blue 71		75			
		Basic Red 9 base		72			
		Reactive Black 5		88			
Trametes hirsuta (THL1 and THL2)	0.2 nkat/ml	Indigo, 1 mg	5 ml vol. in eprouvettes		Isatin and anthranilic acid	120h	Campos et al., 2001
Sclerotium rolfsii (SRL1) THL1 or THL2	+HBT or 4-Hydroxybenzenesulfonic acid or acetosyringone, 10µM			27–31		24h	

TABLE 10.9 *Continued*

Fungus	Laccase Conc./Mediator	Dye Conc.	Medium/Type of Bioreactor	Color Removal (%)	Metabolic Products	Incubation (minutes/hours)	Reference
SRL1	+HBT or 4-Hydroxybenzenesulfonic acid or acetosyringone, 10μM			24–27		24h	
Coriolopsis gallica	1–2–2.3mg/ml	Reactive Blue 198	Immobilized on activated agarose	85		50h	Reyes et al., 1999
Trametes versicolor	162U/l + 0.5μM ABTS	Azo Anthraquinone Indigo		84mg/l per hour 142mg/l per hour 90mg/l per hour			Wong and Yu, 1999
Pleurotus ostreatus strain 32	30U/l 30U/l + 0.16% ABTS	Remazol Brilliant Blue, 100mg/l	10ml vol.	66.3 90		10h 5h	Hou et al., 2004
Aspergillus sp. (genetically modified)	Commercial laccase 10U/ml laccase + 11mM HBT + 5.7mM VA Commercial laccase, 5 or 10U/ml Laccase, 10U/ml + 0.12 or 0.15% HBT	Remazol Brilliant Blue R, 0.02g/l	25ml vol.	30 50 90 100 100		20m 20m 20m 120m 60m	Soares et al., 2001a Soares et al., 2001b
Thelephora sp.	15U/ml	Orange G Congo Red Amido Black 10B	Phosphate buffer, 0.1M	19 12 15			Selvam et al., 2003
Pleurotus ostreatus	POXC/POXA3 mixture (1:1 U:U)	Remazol Brilliant Blue R, 50μM	Sodium acetate buffer, 20mM	74		100m	Palmieri et al., 2005

difference in laccase isozymes and to the difference in specificities to different dyes of diverse structures (Nyanhongo et al., 2002). *T. modesta* laccase has the highest potential for the transformation of dyes to colorless compounds. Purified laccases from *Trametes hirsuta* (THL1 and THL2) and *Sclerotium rolfsii* (SRL1) degraded a textile Indigo dye (Campos et al., 2001). A possible pathway for the laccase-catalyzed oxidation of Indigo dye is shown in Figure 10.5. The catalytic activity of laccases involves the stepwise
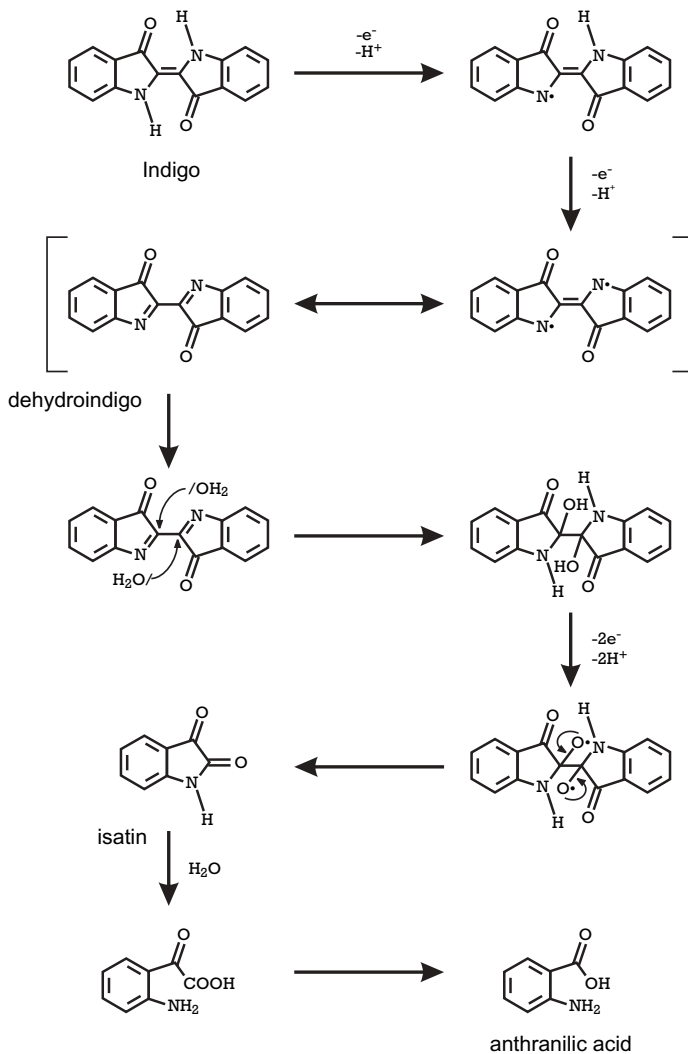
Figure 10.5 Tentative mechanism for laccase-catalyzed degradation of Indigo Carmine dye. [Reprinted from Campos et al. (2001), copyright © with permission from Elsevier.]

abstraction of four electrons from the substrate and proceeds analogously to the electrochemical oxidation of Indigo Carmine, which produces dehydro-indigo. Four electrons are necessary to reduce an O_2 molecule to water and to oxidize Indigo to isatin (indole-2,3-dione) as an intermediate. After the formation of isatin, further degradation occurred hydrolytically without the mediation of laccase. Isatin led to unstable isatic acid as an intermediate that decomposed spontaneously via decarboxylation to yield anthranilic acid (2-aminobenzoic acid) as the final degradation product. Indigo degradation proceeded at a similarly timely rate for both laccases from *T. hirsuta* and occurred at a slower rate for the laccase of *S. rolfsii*. In addition, THL1 and SRL1 laccase treatments reduced the particle size of indigo agglomerates. Incubating indigo-dyed fabrics with laccases from *T. hirsuta* and *S. rolfsii* produced various bleaching effects, and this correlates with the release of indigo degradation products.

Several factors influence the rate of enzymatic degradation. These include adsorption phenomena, transport limitations and accessibility, and the redox potential of the laccase and the substrate. Steric effects may not influence oxidation rates if the binding sites of laccases for the reducing substrate appear to be shallow. For smaller substrates, such as Indigo, the electronic substituents on the aromatic ring are more important than the steric effects. A high redox potential, between 0.4 and 0.8 V, in the laccases will enhance the velocity of electron transfer from the substrate to the T1 site and the oxidation rate. Higher oxidation rates for *T. hirsuta* laccases than for *S. rolfsii* laccase do not suggest a higher redox potential of THL1 and THL2, as this situation may change using other substrates and reaction conditions (Campos et al., 2001). Electron-donating methyl and methoxy substituents appear to enhance laccase activity, while electron-withdrawing chloro, fluoro, and nitro substituents inhibit oxidation of azophenols by fungal laccases.

10.12.2.1   Laccase Bioreactors. Anthraquinone, azo, indigo, and triaryl-methane dyes are degraded by laccase purified from *Trametes hirsuta* (Abadulla et al., 2000b). Initial rates of decolorization depend on the substituents on the phenolic rings of the dyes. Immobilization of the *T. hirsuta* laccase on alumina increases the thermal stability of enzymes and its tolerance against enzyme inhibitors such as halides, copper chelators, and dyeing additives. Up to 80% of the toxicity of anthaquinone dyes is reduced by immobilized laccase. Immobilized laccase from *Coriolopsis gallica* UAMH 8260 retained 85% of the initial activity after 10 cycles lasting 15 hours and 70% after 21 hours over 3 months of intermittent use in decolorization of Reactive Blue 198 dye (Reyes et al., 1999). Free laccase decolorized 13 of 38 industrial dyes and 26 of 38 dyes in the presence of 1 mM HBT as a free radical mediator, enhancing both the range and degree of decolorization. Addition of activators such as ethanol and VA and aeration can increase the laccase levels in *Trametes versicolor* (Maceiras et al., 2001). *T. versicolor* grown on nylon sponge functioning as a physical support for bound mycelium can decolorize the

polymeric dye Poly R-478, and 90% decolorization occurs in VA-supplemented cultures.

10.12.2.2 Influence of Mediators. Several mediators are recognized to enhance the degradation of dyes by laccases. The mechanism of catalysis of laccase is different depending on the dye structure (Wong and Yu, 1999). The Michaelis–Menten kinetics of enzymes implies that the anthraquinone dye is a substrate for laccase. Azo and Indigo dyes are not good substrates, and their degradation requires the mediation of some small-molecule (<8 kDa) metabolites. About 20-fold degradation of azo dyes and Indigo Carmine occurs in the presence of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS). Bleaching of fabrics by laccases in the presence of mediators has been correlated with the release of Indigo degradation products (Campos et al., 2001).

Various mediators show remarkable differences in their abilities to enhance dye decolorization by laccase from *Trametes modesta* (Nyanhongo et al., 2002). Decolorization of Acid Blue 225, Acid Violet 17, and Reactive Black 5 was enhanced two- to sixfold in the presence of HBT at 50ºC. Two mediators, 2-methoxyphenothiazine (MPT) and 3′, 5′-dimethoxy-4-hydroxy-acetophenone (DMHAP), enhanced the decolorization of Basic Red 9 base. On the contrary, mediators such as DMHAP and 4-hydroxy-2,2,6,6-tetramethyl-piperidine (HTMP) inhibited the decolorization of some dyes.

A commercial formulation of pure fungal laccase produced from the submerged fermentation of a genetically modified *Aspergillus* sp. has been employed for the decolorization of Remazol Brilliant Blue R (RBBR) (Soares et al., 2001a). This formulation contains laccase, a mediator (phenothiazine-10-propionic acid), and a nonionic surfactant in phosphate buffer. The commercial laccase formulation (CLF) decolorized 30% of RBBR after 20 minutes. Violuric acid at 5.7 mM concentration with laccase was the most effective mediator. According to the Michaelis–Menten equation, the kinetic parameters of laccase-catalyzed reactions follow pseudo-first-order kinetics. Comparison of fungal laccases in combination with a variety of mediators indicates that the redox potential of the laccases varies depending on the source of the laccase. A wild-type *Coprinus cinereus* laccase and its recombinant form expressed in *Aspergillus oryzae* has been purified and characterized (Schneider et al., 1999). This laccase, mediated by several phenols and phenothiazines, can bleach Direct Blue 1 dye oxidatively at an alkaline pH.

10.13 DECOLORIZATION OF ARTIFICIAL TEXTILE EFFLUENT

During the last decade, some researchers prepared artificial textile dye effluents and used fungi for decolorization and degradation. Such studies are limited and are noted in Table 10.10. Eight individual dyes were decolorized extensively by *P. chrysosporium* (93 to 100%), compared to 74 to 96% for *C. versicolor* in an artificial textile effluent (Kirby et al., 1997). Glucose

TABLE 10.10 Fungal Decolorization of Artificial Textile Effluent

Fungus	Artificial Textile Effluent/ No. of Dyes	Medium	Color Removal/ Change (%)	Enzyme Activity (U/ml)	Incubation (days)	Reference
Phanerochaete chrysosporium	Eight dyes	N-limited cultures	99 Blue → wine → colorless	MnP 85	14 d	Kirby et al., 1997
Coriolus versicolor			98 Blue → wine	MnP 54		
Phlebia tremellosa	Eight dyes, each 200 mg/l	Modified basal medium	96	Laccase 15	14 d	Kirby et al., 2000
		Flushed with O₂	98		14 d	
Aspergillus foetidus	Five dyes, each 10 mg/l	Mineral salts medium	70		2 d	Sumathi and Manju, 2000
P. tremellosa	Five dyes, each 200 mg/l		85		3 d	
		N-rich	79	LiP 180, laccase 390	9 d	Robinson et al., 2001b
		N-limited	74	LiP 115, laccase 218	11 d	
Bjerkandera adusta		N-rich	85	LiP 133, laccase 23	7 d	
		N-limited	86	LiP 113, laccase 13	9 d	
Coriolopsis gallica		N-rich	81	LiP 133, laccase 23	11 d	Robinson et al., 2001c
		N-limited	87	LiP 113, laccase 14	11 d	
P. chrysosporium		N-rich	54	LiP 153, laccase 15	11 d	
		N-limited	48	LiP 77, laccase 13	11 d	
Phanerochaete sordida	Four dyes, each 200 mg/l	N-limited glucose ammonium medium	90	MnP	2 d	Harazono and Nakamura, 2005

consumption was reduced linearly but not depleted completely. The greatest color reduction occurred when maximum MnP levels were detected. Addition of aromatic compounds into the culture medium not only enhanced the activity of MnP two- to fourfold but also changed the isozyme composition (Leontievsky et al., 1991). In an artificial effluent containing eight reactive azo dyes, *Trametes versicolor* showed nearly 90% decolorization after 7 days along with a biomass of 1.20 g/l (Martins et al., 2003). Immobilized *Irpex lacteus* efficiently decolorized a textile effluent containing different dye mixtures (Novotny et al., 2004). Drimarene Blue, Drimarene Red, Remazol Green, and Acid Black decolorized to 100%, 80%, 45%, and 35%, respectively, within 3 to 5 days. *Phlebia tremellosa* removed 98% of the color of an artificial textile effluent containing nine dyes after 14 days when flushed daily with oxygen (Kirby et al., 2000). In addition to laccase, another process appears to be responsible for color removal in the absence of detectable levels of this enzyme.

Aspergillus foetidus decolorized a mixture of dyes simultaneously without preference (Sumathi and Manju, 2000). This implies the presence of a nonspecific system vital for the treatment of textile effluents. *Geotrichum* sp. CCMI 1019 transformed all three reactive dyes in 5 days when 20-day-old cultures were spiked with successive amounts (200 ppm) of dyes (Maximo et al., 2003). The fungus has the sustained ability to transform a large amount of dyes (800 ppm), suggesting potential applications in decolorization of the textile wastewater. In an artificial textile effluent of five dyes, nitrogen addition had certain effects: improved the enzyme activities and dye decolorization due to *Phanerochaete chrysosporium*; increased the enzyme activities for *Coriolopsis gallica* without improvement in decolorization; and had no major effect on dye degradation due to *Bjerkandera adusta* but with a slight increase by *Phlebia tremellosa* (Robinson et al., 2001b,c). The highest levels of LiP and laccase were produced in the presence of dyes in nitrogen-rich media, almost twice the levels of nitrogen-limited media. The LiP activity doubled and laccase activity quadrupled for *P. tremellosa* in nitrogen-rich media. *B. adusta* shows lower enzyme activities in nitrogen-rich media. MnP activities are low and it is difficult to assess their role in dye decolorization.

Industrial effluents contain a number of dyes, and some of them may be anthraquinone dyes, which are good substrates of laccase. Azo dye can be degraded by repeated additions of anthraquinone dyes (Wong and Yu, 1999). The slow degradation of nonsubstrate azo and indigo dyes by laccase increased at a low concentration of anthraquinone dye. Anthraquinone dye acted as a mediator instead of a catalyst, as it also decomposed gradually with the decomposition of other dyes. Immobilized laccase from *Coriolopsis gallica* decolorized nearly 100% of an artificial textile effluent containing Direct Blue 200, Direct Red 80, Direct Black 22, sodium sulfate, sodium bicarbonate, soap, and dispersant within 120 hours (Reyes et al., 1999). Immobilized laccase retained only 14% of initial activity after 10 cycles.

10.14   SEQUENTIAL DYE DECOLORIZATION

Little is known at present on sequential dye decolorization. Aqueous sequential decolorization occurred in batch cultures of white-rot fungi in a 20-day period (Swamy and Ramsay, 1999a). The addition of each dye was in the order Amaranth, Remazol Black, Remazol Orange, Tropaeolin O, Reactive Blue, Remazol Black, and Remazol Orange, and the dye was added only after previous complete dye decolorization. *Trametes versicolor* maintained a high level of color removal on sequential additions of various dyes, including a mixture of Remazol Black, Remazol Orange, and Reactive Blue. The physiological differences among three cultures may account for the differences in decolorizing enzymes and eventually, the decolorization ability. All three fungi are known to secrete LiP and MnP, but *T. versicolor* also produces laccases.

Mycelial pellets of *T. versicolor* decolorized successive additions of Amaranth and of different dyes and dye mixtures containing glucose and ammonium tartrate (Swamy and Ramsay, 1999b). Constant rates of decolorization were sustained above $0.13\,g/l$ of glucose. Decolorization followed first-order kinetics for Amaranth concentration. It is possible that glucose is a substrate for the generation of H_2O_2 required for extracellular peroxidase activity and/or the generation of Mn(III)-complexing agents necessary for MnP activity. Rapid color removal was maintained at $0.086\,g/l\,NH_4^+$. The culture conditions of repressing ligninolysis and dye decolorization by *P. chrysosporium* indicate that dye decolorization by *T. versicolor* may be a distinct process from that of *P. chrysosporium*.

10.15   CONCLUSIONS AND FUTURE PERSPECTIVES

Color-free effluent discharge to receiving waters is a main concern of the public at all times. Decolorization of the complicated dye molecules, salt concentrations, and a wide range of pH cause many complications. The limitations of physicochemical processes lead to a search for new methods of dye decolorization. This approach involves several aspects related to decolorization, degradation, and mineralization of dyes. This process is fungal, economical, unique, and innovative to industry and has public acceptance. Various bioreactors are clearly indicators of success in the use of fungi in textile and dyestuff-containing wastewaters. At present, computer models are unknown in fungal dye decolorization technology. However, models can predict the performance of bioreactors under various operating conditions and clearly add valuable data on the dynamics of the system. By optimizing the conditions and requirements, including the coproduction of relevant enzymes, H_2O_2, and VA, a cost-effective and reliable configured bioreactor can be established, resulting in a high rate of decolorization and range of dye compounds.

A considerable amount of work has been done dealing with the screening of fungal genera and species in the decolorization and degradation of dye-containing wastewaters. At present, most screening studies are based on the wood-rotting basidiomycete fungi. The ability of white-rot fungi to degrade dyes implies widespread decolorizing ability among fungi. We should employ a screening system to other classes of fungi to discover new, effective, dye-decolorizing fungal species superior to *P. chrysosporium* that do not produce unwanted side products. Nearly 77% of 150 µg/g of Remazol Brilliant Blue R was removed from a soil environment by *Irpex lacteus* after 7 days at 28ºC (Novotny et al., 2001). Thermophilic or thermotolerant fungi have not been exploited in dye decolorization systems. Furthermore, a search for fungal species that utilize dyes as a sole source of carbon may produce future applications. Also, exploration of a new class of enzymes that degrade dyes should be continued to maximize the benefit of these organisms.

Textile wastewater always contains a wide range and concentration of dyes. Successful decoloration of a single dye does not effectively suggest the suitability of a fungus in a decoloration treatment system. This implies the development of an appropriate biodecoloration system that can handle and sustain a high level of activity upon repeated exposure to dyes. Little is known of the fungal treatment of mixtures of dyes and sequential decolorization, but fungi have a great potential to degrade textile effluents. Sequential decolorization demands a step-by-step examination of media and extracellular contents. In certain cases, industrial effluents contain substrate dyes that can induce the degradation of nonsubstrate dyes. Certain compounds can also mediate the degradation of nonsubstrate dyes. More research is needed to establish the relationship between the molecular structure of dyes and fungal decolorization.

Variations in the composition of effluents define the need for a nonspecific process for treatment. Use of the nonspecific ligninolytic system by white-rot fungi to decolorize dyes of structurally different classes has been well demonstrated. Fungal enzymes such as LiP, MnP, and laccases can cleave aromatic rings and have the potential to break down dye molecules. Dye degradation relationship among mediators, nonsubstrate dyes, and laccase can open new horizons in the biodegradation of various dyes in industrial effluents. There have been several instances where dyes were decolorized by unknown enzymes or mechanisms. An elucidation of the mechanisms and metabolic pathways will facilitate the development of novel types of bioreactors.

Molecular biology and biotechnology have not played an active role in the genetic manipulation of fungi for the biodegradation of dye-containing wastewater. Fungal genes have not been isolated that are specific for the biodegradation of dye-containing wastewater. However, several genes encoding for different isozymes of LiP have been isolated and sequenced. A future exists for the development of genes cloning for the decolorization and degradation of dyes as well as bioengineered fungi that can utilize dyes as sole substrates.

REFERENCES

Abadulla, E., K.-H. Robra, G.M. Gubitz, L.M. Silva, and A. Cavaco-Paulo (2000a) Enzymatic decolorization of textile dyeing effluents. *Text. Res. J.* **70**: 409–414.

Abadulla, E., T. Tzanov, S. Costa, K.-H. Robra, A. Cavaco-Paulo, and G.M. Gubitz (2000b) Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta. Appl. Environ. Microbiol.* **66**: 3357–3362.

Aksu, Z. (2003) Reactive dye accumulation by *Saccharomyces cerevisiae. Process Biochem.* **38**: 1437–1444.

Aksu, Z., and G. Donmez (2003) A comparative study on the biosorption characteristics of some yeasts for Remazol Blue reactive dye. *Chemosphere* **50**: 1075–1083.

Ambrosio, S.T., and G.M. Campos-Takaki (2004) Decolorization of reactive azo dyes by *Cunninghamella elegans* UCP 542 under co-metabolic conditions. *Bioresour. Technol.* **91**: 69–75.

An, H., Y. Qian, X. Gu, and W.Z. Tang (1996) Biological treatment of dye wastewaters using an anaerobic–oxic system. *Chemosphere* **33**: 2533–2542.

Angelis, D.F., and G.S. Rodrigues (1987) Azo dyes removal from industrial effluents using yeast biomass. *Arq. Biol. Tecnol.* **30**: 301–309.

APHA (1995) *Standard Methods for Examination of Water and Wastewater*, 19th ed. American Public Health Association, Washington, DC.

Arslan, I., and I.A. Bacioglu (1999) Degradation of commercial reactive dyestuffs by heterogeneous and homogeneous advanced oxidation processes: a comparative study. *Dyes Pigm.* **43**: 95–108.

Assadi, M.M., and M.Z. Jahangiri (2001) Textile wastewater treatment by *Aspergillus niger. Desalination* **141**: 1–6.

Azmi, W., R.K. Sani, and U.C. Banerjee (1998) Biodegradation of triphenylmethane dyes. *Enzyme Microb. Technol.* **22**: 185–191.

Balan, D.S., and R.T. Monteiro (2001) Decolorization of textile indigo dye by ligninolytic fungi. *J. Biotechnol.* **89**: 141–145.

Banat, I.M., P. Nigam, D. Singh, and R. Marchant (1996) Microbial decolorization of textile-dye-containing effluents: a review. *Bioresour. Technol.* **58**: 217–227.

Barbosa, A.M., R.F.H. Dekker, and G.E. St. Hardy (1996) Veratryl alcohol as an inducer of laccase by an ascomycete, *Botryosphaeria* sp., when screened on the polymeric dye Poly R-478. *Lett. Appl. Microbiol.* **23**: 93–96.

Blanquez, P., N. Casas, X. Font, X. Gabarrell, M. Sarra, et al. (2004) Mechanism of textile metal dye biotransformation by *Trametes versicolor. Water Res.* **38**: 2166–2172.

Borchert, M., and J.A. Libra (2001) Decolorization of reactive dyes by the white rot fungus *Trametes versicolor* in sequencing batch reactors. *Biotechnol. Bioeng.* **75**: 313–321.

Brahimi-Horn, M.C., K.K. Lim, S.L. Liany, and D.G. Mou (1992) Binding of textile azo dyes by *Myrothecium verrucaria*: Orange II, 10B (blue), and RS (red) azo dye uptake for textile wastewater decolorization. *J. Ind. Microbiol.* **10**: 245–261.

Brown, M.A., and S.C. DeVito (1993) Predicting azo dye toxicity. *Crit. Rev. Environ. Sci. Technol.* **23**: 249–324.

Buckley, K.F., and A.D.W. Dobson (1998) Extracellular ligninolytic enzyme production and polymeric dye decolourization in immobilized cultures of *Chrysosporium lignorum* CL1. *Biotechnol. Lett.* **20**: 301–306.

Bumpus, J.A. (1995) Microbial degradation of azo dye. In: *Biotransformations: Microbial Degradation of Health Risk Compounds*, V.P. Singh, ed. Elsevier, New York, pp. 157–176.

Bumpus, J.A., and B.J. Brock (1988) Biodegradation of crystal violet by white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **54**: 1143–1149.

Bustard, M., G. McMullan, and A.P. McHale (1998) Biosorption of textile dyes by biomass derived from *Kluyveromyces marxianus* IMB3. *Bioprocess Eng.* **19**: 427–430.

Campos, R., A. Kandelbauer, K.H. Robra, A. Cavaco-Paulo, and G.M. Gubitz (2001) Indigo degradation with purified laccases from *Trametes hirsuta* and *Sclerotium rolfsii*. *J. Biotechnol.* **89**: 131–139.

Capalash, N., and P. Sharma (1992) Biodegradation of textile azo-dyes by *Phanerochaete chrysosporium*. *World J. Microbiol. Biotechnol.* **8**: 309–312.

Cha, C.-J., D.R. Doerge, and C.E. Cerniglia (2001) Biotransformation of malachite green by the fungus *Cunninghamella elegans*. *Appl. Environ. Microbiol.* **67**: 4358–4360.

Chagas, E.P., and L.R. Durrant (2001) Decolorization of azo dyes by *Phanerochaete chrysosporum* and *Pleurotus sajorcaju*. *Enzyme Microb. Technol.* **29**: 473–477.

Chao, W.L., and S.L. Lee (1994) Decolorization of azo dyes by three white-rot fungi: influence of carbon source. *World J. Microbiol. Biotechnol.* **10**: 556–559.

Chivukula, M., and V. Renganathan (1995) Phenolic azo dye oxidation by laccase by *Pyricularia oryzae*. *Appl. Environ. Microbiol.* **61**: 4374–4377.

Chivukula, M., J.T. Spadaro, and V. Renganathan (1995) Lignin peroxidase-catalyzed oxidation of sulfonated azo dyes generates novel sulfophenyl hydroperoxides. *Biochemistry* **334**: 7765–7772.

Christian, V., R. Shrivastava, D. Shukla, H. Modi, and B.R.M. Vyas (2005) Mediator role of veratryl alcohol in the lignin peroxidase-catalyzed oxidative decolorization of Remazol Brilliant Blue R. *Enzyme Microb. Technol.* **36**: 426–431.

Churchley, J.H. (1994) Removal of dye wastewater color from sewage effluent: the use of a full scale ozone plant. *Water Sci. Technol.* **30**: 275–284.

Conneely, A., W.F. Smyth, and G. McMullan (1999) Metabolism of the phthalocyanine textile dye remazol turquoise blue by *Phanerochaete chrysosporium*. *FEMS Microbiol. Lett.* **179**: 333–337.

Cripps, C., J.A. Bumpus, and S.D. Aust (1990) Biodegradation of azo and heterocyclic dyes by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **56**: 1114–1118.

Das, S.S., S. Dey, and B.C. Bhattacharyya (1995) Dye decolourization in column bioreactor using the wood-degrading fungus *Phanerochaete chrysosporium*. *Ind. Chem. Eng.* **37**: 176–180.

DEPA (1998) *Azocolorants in Textiles and Toys*. No. 4161998. Danish Environmental Protection Agency, Copenhagen, Denmark. www.mst.dk/199812publikat/87-7909-136-9/indhold_eng.htm.

Dey, S., T.K. Maiti, and B.C. Bhattacharyya (1994) Production of some extracellular enzymes by a lignin peroxidase-producing brown rot fungus, *Polyporus ostreiformis*, and its comparative abilities for lignin degradation and dye decolorization. *Appl. Environ. Microbiol.* **60**: 4216–4218.

Dominguez, A., I. Rivela, S.R. Couto, and M.A. Sanroman (2001) Design of a new rotating drum bioreactor for ligninolytic enzyme production by *Phanerochaete chrysosporium* grown on an inert support. *Process Biochem.* **37**: 549–554.

ECT (1977) *Kirk–Othmer Encyclopedia of Chemical Technology*, 3rd ed. Wiley, New York.

Eichlerova, I., L. Homolka, L. Lisa, and F. Nerud (2005) Orange G and Remazol Brilliant Blue R decolorization by white rot fungi *Dichomitus squalens*, *Ischnoderma resinosum* and *Pleurotus calyptratus*. *Chemosphere* **60**: 398–404.

Ferreira, V.S., D.B. Magalhaes, S.H. Kling, J.G. da Silva, Jr., and E.P.S. Bon (2000) N-demethylation of Methylene Blue by lignin peroxidase from *Phanerochaete chrysosporium*. *Appl. Biochem. Biotechnol.* **84–86**: 255–265.

Field, J.A., F. Boelsma, H. Baten, and W.H. Rulkens (1995) Oxidation of anthracene in water/solvent mixtures by the white-rot fungus *Bjerkandera* sp. strain BOS55. *Appl. Microbiol. Biotechnol.* **44**: 234–240.

Fitzgerald, S.W., and P.L. Bishop (1995) Two stage anaerobic/aerobic treatment of sulfonated azo dyes. *J. Environ. Sci. Health A* **30**: 1251–1276.

Fu, Y., and T. Viraraghavan (2001) Fungal decolorization of dye wastewaters: a review. *Bioresour. Technol.* **79**: 251–262.

Fu, Y., and T. Viraraghavan (2002) Removal of Congo Red from an aqueous solution by fungus *Aspergillus niger*. *Adv. Environ. Res.* **7**: 239–247.

Gahr, F., F. Hermanutz, and W. Oppermann (1994) Ozonation: an important technique to comply with new German laws for textile wastewater treatment. *Water Sci. Technol.* **30**: 255–263.

Ganesh, R., G.D. Boardman, and D. Michelsen (1994) Fate of azo dyes in sludges. *Water Res.* **28**: 1367–1376.

Ge, Y., L. Yan, and K. Qinge (2004) Effect of environment factors on dye decolorization by *P. sordida* ATCC 90872 in a aerated reactor. *Process Biochem.* **39**: 1401–1405.

Glenn, J.K., and M.H. Gold (1983) Decolorization of several polymeric dyes by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **45**: 1741–1747.

Gogna, E., R. Vohra, and P. Sharma (1992) Biodegradation of Rose Bengal by *Phanerochaete chrysosporium*. *Lett. Appl. Microbiol.* **14**: 58–60.

Goszczynski, S., A. Paszczynski, M.B. Pasti-Grigsby, R.L. Crawford, and D.L. Crawford (1994) New pathway for the degradation of sulfonated azo dyes by microbial peroxidases of *Phanerochaete chrysosporium* and *Streptomyces chromofuscus*. *J. Bacteriol.* **176**: 1339–1347.

Hao, O.J., H. Kim, and P.-C. Chiang (2000) Decolorization of wastewater. *Crit. Rev. Environ. Sci. Technol.* **30**: 449–505.

Harazono, K., and K. Nakamura (2005) Decolorization of mixtures of different reactive textile dyes by the white-rot basidiomycetes *Phanerochaete sordida* and inhibitory effect of polyvinyl alcohol. *Chemosphere* **59**: 63–68.

Hardin, I.R., H. Cao, S.S. Wilson, and D.E. Akin (2000) Decolorization of textile wastewater by selective fungi. *Text. Chem. Colorist Am. Dyestuff Rep.* **32**: 38–42.

Harmer, C., and P. Bishop (1992) Transformation of azo dye AO-7 by wastewater biofilms. *Water Sci. Technol.* **26**: 627–636.

Heinfling, A., M. Bergbauer, and U. Szewzyk (1997) Biodegradation of azo and phthalocyanine dyes by *Trametes versicolor* and *Bjerkandera adusta*. *Appl. Microbiol. Biotechnol.* **48**: 261–266.

Heinfling, A., M.J. Martinez, A.T. Martinez, M. Bergbauer, and U. Szewzyk (1998) Transformation of industrial dyes by manganese peroxidases from *Bjerkandera adusta* and *Pleurotus eryngii* in a manganese-independent reaction. *Appl. Environ. Microbiol.* **64**: 2788–2793.

Heinfling-Weidtmann, A., T. Reemtsma, T. Storm, and U. Szewzyk (2001) Sulfophthalimide as major metabolite formed from sulfonated phthalocyanine dyes by the white rot fungus *Bjerkandera adusta*. *FEMS Microbiol. Lett.* **203**: 179–183.

Ho, Y.S., D.A.J. Wase, and C.F. Forster (1996) Kinetic studies of competitive heavy metal adsorption by sphagnum moss peat. *Environ. Technol.* **17**: 71–77.

Hou, H., J. Zhou, J. Wang, C. Du, and B. Yan (2004) Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. *Process Biochem.* **39**: 1415–1419.

Hu, T.L. (1994) Decolorization of reactive dyes by transformation with *Pseudomonas luteola*. *Bioresour. Technol.* **49**: 47–51.

Ingamells, W. (1993) *Colour for Textiles: A User's Handbook*. Society of Dyers and Colourists, West Yorkshire, UK.

Jarosz-Wilkolazka, A., J. Kochmanska-Rdest, E. Malarczyk, W. Wardas, and A. Leonowicz (2002) Fungi and their ability to decolourize azo and anthraquinone dyes. *Enzyme Microb. Technol.* **30**: 566–572.

Jiang, H., and P.L. Bishop (1994) Aerobic biodegradation of azo dyes in biofilms. *Water Sci. Technol.* **29**: 525–530.

Jinqi, L., and L. Houtian (1992) Degradation of azo dyes by algae. *Environ. Pollut.* **75**: 273–278.

Kapdan, I.K., and F. Kargi (2002a) Biological decolorization of a textile dyestuff containing wastewater by *Coriolus versicolor* in a rotating biological contactor. *Enzyme Microb. Technol.* **30**: 195–199.

Kapdan, I.K., and F. Kargi (2002b) Simultaneous biodegradation and adsorption of textile dyestuff in an activated sludge unit. *Process Biochem.* **37**: 973–981.

Kapdan, I.K., and R. Oztekin (2003) Decolorization of textile dyestuff Reactive Orange 16 in fed-batch reactor under anaerobic condition. *Enzyme Microb. Technol.* **33**: 231–235.

Kapdan, I.K., F. Kargi, G. McMullan, and R. Marchant (2000) Effect of environmental conditions on biological decolorization of textile dyestuff by *C. versicolor*. *Enzyme Microb. Technol.* **26**: 381–387.

Kasinath, A., C. Novotny, K. Svobodova, K.C. Patel, and V. Sasek (2003) Decolorization of synthetic dyes by *Irpex lacteus* in liquid cultures and packed-bed bioreactor. *Enzyme Microb. Technol.* **32**: 167–173.

Kennedy, M.T., J.M. Morgan, L.K. Benefield, and A.F. McFadden (1992) Color removal from textile dye wastewater: a case study. In: *Proceedings of the 47th Industrial Waste Conference*, West Lafayette, IN. Lewis Publishers, Chelsea, MI, pp. 727–741.

Khehra, M.S., H.S. Saini, D.K. Sharma, et al. (2006) Biodegradation of azo dye C. I. Acid Red 88 by an anoxic–aerobic sequential bioreactor. *Dyes Pigm.* **70**: 1–7.

Kim, S.J., and M. Shoda (1999a) Decolorization of molasses and a dye by a newly isolated strain of the fungus *Geotrichum candidum* Dec 1. *Biotechnol. Bioeng.* **62**: 114–119.

Kim, S.J., and M. Shoda (1999b) Purification and characterization of a novel peroxidase from *Geotrichum candidum* Dec 1 involved in decolorization of dyes. *Appl. Environ. Microbiol.* **65**: 1029–1035.

Kim, T.-H., Y. Lee, J. Yang, B. Lee, C. Park, and S. Kim (2004) Decolorization of dye solutions by a membrane bioreactor (MBR) using white-rot fungi. *Desalination* **168**: 287–293.

Kirby, N., G. McMullan, and R. Marchant (1997) Bioremediation of textile industry by white-rot fungi. In: *Global Environmental Biotechnology*, D.L. Wise, ed. Elsevier, New York, pp. 711–718.

Kirby, N., R. Marchant, and G. McMullan (2000) Decolourization of synthetic textile dyes by *Phlebia tremellosa*. *FEMS Microbiol. Lett.* **188**: 93–96.

Knapp, J.S., and P.S. Newby (1999) The decolourization of a chemical industry effluent by white rot fungi. *Water Res.* **33**: 575–577.

Knapp, J.S., P.S. Newby, and L.P. Reece (1995) Decolorization of dyes by wood-rotting basidiomycete fungi. *Enzyme Microb. Technol.* **17**: 664–668.

Knapp, J.S., F. Zhang, and K.N. Tapley (1997) Decolourization of Orange II by a wood-rotting fungus. *J. Chem. Technol. Biotechnol.* **69**: 289–296.

Knapp, J.S., E.J. Vantoch-Wood, and F. Zhang (2001) Use of wood-rotting fungi for the decolorization of dyes and industrial effluents. In: *Fungi in Bioremediation*. G.M. Gadd, ed. Cambridge University Press, Cambridge, pp. 242–304.

Kothandaraman, U., K.M. Aboo, and C. Sastry (1976) Characteristics of wastes from a textile mill. *Indian J. Environ. Health* **18**: 99–112.

Kunz, A., V. Reginatto, and N. Duran (2001) Combined treatment of textile effluent using the sequence *Phanerochaete chrysosporium*-ozone. *Chemosphere* **44**: 281–287.

Kwasniewska, K. (1985) Biodegradation of crystal violet (hexamethyl-p-rosaniline chloride) by oxidation of red yeasts. *Bull. Environ. Contam. Toxicol.* **34**: 323–330.

Leidig, E., U. Prusse, K.D. Vorlop, and J. Winter (1999) Biotransformation of Poly R-478 by continuous cultures of PVAL-encapsulated *Trametes versicolor* under non-sterile conditions. *Bioprocess Eng.* **21**: 5–12.

Leontievsky, A.A., N.M. Ayasoedova, E.J. Kolomiyers, and L.A. Golovleva (1991) Induction of ligninolytic enzymes of the white-rot fungus *Panus tigrinus*. *Biochemistry* (*Moscow*) **56**: 1173–1180.

Levin, L., A. Jordan, F. Forchiassin, and A. Viale (2001) Degradation of anthraqui-none blue by *Trametes trogii. Rev. Argent. Microbiol.* **33**: 223–228.

Levin, L., L. Papinutti, and F. Forchiassin (2004) Evaluation of Argentinean white-rot fungi for their ability to produce lignin-modifying enzymes and decolorize industrial dyes. *Bioresour. Technol.* **94**: 169–176.

Lopez, C., I. Mielgo, M.T. Moreira, G. Feijoo, and J.M. Lema (2002) Enzymatic membrane reactors for biodegradation of recalcitrant compounds. *J. Biotechnol.* **99**: 249–257.

Lowry, G.G., and R.C. Lowry (1988) *Lowry's Handbook of Right to Know and Emergency Planning.* Lewis Publishers, Chelsea, MI, pp. 332–382.

Loyd, C.K., G.D. Boardman, and D.L. Michelsen (1992) Anaerobic/aerobic treatment of a textile dye wastewater. *Hazard. Ind. Wastes* **24**: 593–601.

Maceiras, R., S. Rodriguez-Couto, and A. Sanroman (2001) Influence of several activators on the extracellular laccase activity and in vivo decolourization of Poly R-478 by semi-solid-state cultures of *Trametes versicolor. Acta Biotechnol.* **21**: 255–264.

Marchant, R., P. Nigam, and I.M. Banat (1994) An unusual facultatively anaerobic fungus isolated from prolonged enrichment culture conditions. *Mycol. Res.* **98**: 757–760.

Martins, M.A.M., M.H. Cardoso, M.J. Queiroz, M.T. Ramalho, and A.M.O. Campos (1999) Biodegradation of azo dyes by the yeast *Candida zeylanoides* in batch aerated cultures. *Chemosphere* **38**: 2455–2460.

Martins, M.A.M., I.C. Ferreira, I.M. Santos, M.J. Queiroz, and N. Lima (2001) Biodegradation of bioaccessible textile azo dyes by *Phanerochaete chrysosporium. J. Biotechnol.* **89**: 91–98.

Martins, M.A.M., N. Lima, A.J.D. Silvestre, and M.J. Queiroz (2003) Comparative studies of fungal biodegradation of single or mixed bioaccessible reactive azo dyes. *Chemosphere* **52**: 967–973.

Mass, R., and S. Chaudhari (2005) Adsorption and biological decolourization of azo dye Reactive Red 2 in semicontinuous anaerobic reactors. *Process Biochem.* **40**: 699–705.

Maurya, N.S., A.K. Mittal, P. Cornel, and E. Rother (2006) Biosorption of dyes using dead macro fungi: effect of dye structure, ionic strength and pH. *Bioresour. Technol.* **97**: 512–521.

Maximo, C., and M. Costa-Ferreira (2004) Decolourization of reactive textile dyes by *Irpex lacteus* and lignin modifying enzymes. *Process Biochem.* **39**: 1475–1479.

Maximo, C., M.T.P. Amorim, and M. Costa-Ferreira (2003) Biotransformation of industrial reactive azo dyes by *Geotrichum* sp. CCMI 1019. *Enzyme Microb. Technol.* **32**: 145–151.

Mazmanci, M.A., and A. Unyayar (2005) Decolourization of Reactive Black 5 by *Funalia trogii* immobilized on *Luffa cylindrica* sponge. *Process Biochem.* **40**: 337–342.

McCurdy, M.W., G.D. Boardman, D.L. Michelsen, and B.M. Woodby (1992) Chemical reduction and oxidation combined with biodegradation for the treatment of a textile dye. In: *Proceedings of the 46th Purdue Industrial Waste Conference.* Lewis Publishers, Chelsea, MI, pp. 229–234.

Meehan, C., I.M. Banat, G. McMullan, P. Nigam, F. Smyth, and R. Marchant (2000) Decolorization of Remazol Black-B using a thermotolerant yeast, *Kluyveromyces marxianus* IMB3. *Environ. Int.* **26**: 75–79.

Mielgo, I., M.T. Moreira, G. Feijoo, and J.M. Lema (2001) A packed-bed fungal bioreactor for the continuous decolourisation of azo-dyes (Orange II). *J. Biotechnol.* **89**: 99–106.

Mielgo, I., M.T. Moreira, G. Feijoo, and J.M. Lema (2002) Biodegradation of a polymeric dye in a pulsed bed bioreactor by immobilized *Phanerochaete chrysosporium*. *Water Res.* **36**: 1896–1901.

Mishra, G., and M. Tripathy (1993) A critical review of the treatments of decolorization of textile effluent. *Colourage* **40**: 35–38.

Moldes, D., S.R. Couto, C. Cameselle, and M.A. Sanroman (2003) Study of the degradation of dyes by MnP of *Phanerochaete chrysosporium* produced in a fixed-bed bioreactor. *Chemosphere* **51**: 295–303.

Moreira, M.T., I. Mielgo, G. Feijoo, and J.M. Lema (2000) Evaluation of different fungal strains in the decolorization of synthetic dyes. *Biotechnol. Lett.* **22**: 1499–1503.

Mou, D.-G., K.K. Lim, and H.P. Shen (1991) Microbial agents for decolorization of dye wastewater. *Biotechnol. Adv.* **9**: 613–622.

Nigam, P., I.M. Banat, D. Oxspring, R. Marchant, D. Singh, and W.F. Smyth (1995a) A new facultative anaerobic filamentous fungus capable of growth on recalcitrant textile dyes as sole carbon source. *Microbios* **84**: 171–185.

Nigam, P., D. Singh, and R. Marchant (1995b) An investigation of the biodegradation of textile dyes by aerobic and anaerobic microorganisms. In: *Environmental Biotechnology: Principles and Practices*, M. Moo-Young, ed. Kluwer, Dordrecht, The Netherlands, pp. 278–292.

Novotny, C., B. Rawal, M. Bhatt, M. Patel, V. Sasek, and H.P. Molitoris (2001) Capacity of *Irpex lacteus* and *Pleurotus ostreatus* for decolorization of chemically different dyes. *J. Biotechnol.* **89**: 113–122.

Novotny, C., K. Svobodova, A. Kasinath, and P. Erbanova (2004) Biodegradation of synthetic dyes *by Irpex lacteus* under various growth conditions. *Int. Biodeterior. Biodegrad.* **54**: 215–223.

Nyanhongo, G.S., J. Gomes, G.M. Gubitz, R. Zvauya, J. Read, and W. Steiner (2002) Decolorization of textile dyes by laccases from a newly isolated strain of *Trametes modesta*. *Water Res.* **36**: 1449–1456.

Ollikka, P., K. Alhonmaki, V.-M. Leppanen, T. Glumoff, T. Raijoli, and I. Suominen (1993) Decolorization of azo, triphenyl methane, heterocyclic, and polymeric dyes by lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **59**: 4010–4016.

Oxspring, D.A., G. McMullan, W.F. Smyth, and R. Marchant (1986) Decolourization and metabolism of the reactive textile dye, Remazol Black B, by an immobilized microbial consortium. *Biotechnol. Lett.* **18**: 527–530.

Palma, C., M.T. Moreira, I. Mielgo, G. Feijoo, and J.M. Lema (1999) Use of a fungal bioreactor as a pretreatment or post-treatment step for continuous decolorization of dyes. *Water Sci. Technol.* **40**: 131–136.

Palmieri, G., G. Cennamo, and G. Sannia (2005) Remazol Brilliant Blue R deco-lourization by the fungus *Pleurotus ostreatus* and its oxidative enzyme system. *Enzyme Microb. Technol.* **36**: 17–24.

Papinutti, V.L., and F. Forchiassin (2004) Modification of malachite green by *Fomes sclerodermeus* and reduction of toxicity to *Phanerochaete chrysosporium*. *FEMS Microbiol. Lett.* **231**: 205–209.

Paszczynski, A., M.B. Pasti, S. Goszczynski, D.L. Crawford, and R.L. Crawford (1991) New approach to improve degradation of recalcitrant dyes by *Streptomyces* spp. and *Phanerochaete chrysosporium*. *Enzyme Microb. Technol.* **13**: 378–384.

Paszczynski, A., M.B. Pasti-Grigsby, S. Goszczynski, R.L. Crawford, and D.L. Craw-ford (1992) Mineralization of sulfonated azo dyes and sulfanilic acid by *Phanero-chaete chrysosporium* and *Streptomyces chromofuscus*. *Appl. Environ. Microbiol.* **58**: 3598–3604.

Pazarlioglu, N.K., R.O. Urek, and F. Ergun (2005) Biodecolourization of Direct Blue 15 by immobilized *Phanerochaete chrysosporium*. *Process Biochem.* **40**: 1923–1929.

Peralta-Zamora, P., A. Kunz, S.G. de Moraes, R. Pelegrini, et al. (1999) Degradation of reactive dyes. I. A comparative study of ozonation, enzymatic and photochemi-cal processes. *Chemosphere* **38**: 835–852.

Podgornik, H., L. Bradesko, and A. Perdih (1995) Transformation of different dyes by extracellular LiPs from *Phanerochaete chrysosporium*. *Prehrambeno-Tehnol. Biotehnol. Rev.* **33**: 79–83.

Podgornik, H., I. Grgic, and A. Perdih (1999) Decolorization rate of dyes using lignin peroxidases of *Phanerochaete chrysosporium*. *Chemosphere* **38**: 1353–1359.

Pointing, S.B., and L.L.P. Vrijmoed (2000) Decolorization of azo and triphenylmeth-ane dyes by *Pycnosporus sanguineus* producing laccase as the sole phenoloxidase. *World J. Microbiol. Biotechnol.* **16**: 317–318.

Radha, K.V., I. Regupathi, A. Arunagiri, and T. Murugesan (2005) Decolorization studies of synthetic dyes using *Phanerochaete chrysosporium* and their kinetics. *Process Biochem.* **40**: 3337–3345.

Raghukumar, C. (2000) Fungi from marine habitats: an application in bioremedia-tion. *Mycol. Res.* **104**: 1222–1226.

Rajaguru, P., K. Kalaiselvi, M. Palanivel, and V. Subburam (2000) Biodegradation of azo dyes in a sequential anaerobic–aerobic system. *Appl. Microbiol. Biotechnol.* **54**: 268–273.

Ramalho, P.A., H. Scholze, M.H. Cardoso, M.T. Ramalho, and A.M. Oliveira-Campos (2002) Improved conditions for the aerobic reductive decolourisation of azo dyes by *Candida zeylanoides*. *Enzyme Microb. Technol.* **31**: 848–854.

Reyes, P., M.A. Pickard, and R. Vazquez-Duhalt (1999) Hydroxybenzotriazole increases the range of textile dyes decolorized by immobilized laccase. *Biotechnol. Lett.* **21**: 875–880.

Robinson, T., G. McMullan, R. Marchant, and P. Nigam (2001a) Remediation of dyes in textile effluent: a critical review on current treatment technologies with a pro-posed alternative. *Bioresour. Technol.* **77**: 247–255.

Robinson, T., B. Chandran, and P. Nigam (2001b) Studies on the production of enzymes by white-rot fungi for the decolourization of textile dyes. *Enzyme Microb. Technol.* **29**: 575–579.

Robinson, T., B. Chandran, and P. Nigam (2001c) Studies on the decolourization of an artificial textile-effluent by white-rot fungi in N-rich and N-limited media. *Appl. Microbiol. Biotechnol.* **57**: 810–814.

Rodriguez, E., M.A. Pickard, and R. Vazquez-Duhalt (1999) Industrial dye decolorization by laccases from ligninolytic fungi. *Curr. Microbiol.* **38**: 27–32.

Rodriguez Couto, S., I. Rivela, and A. Sanroman (2000) In vivo decolourization of the polymeric dye Poly R-478 by corncob cultures of *Phanerochaete chrysosporium*. *Acta Biotechnol.* **20**: 31–38.

Rozzi, A., F. Malpei, L. Bonomo, and R. Bianchi (1999) Textile wastewater reuse in northern Italy (COMO). *Water Sci. Technol.* **39**: 121–128.

Ryu, B.-H., and Y.D. Weon (1992) Decolorization of azo dyes by *Aspergillus sojae* B-10. *J. Microbiol. Biotechnol.* **2**: 215–219.

Safarikova, M., L. Ptackova, I. Kibrikova, and I. Safarik (2005) Biosorption of water-soluble dyes on magnetically modified *Saccharomyces cerevisiae* subsp. *uvarum* cells. *Chemosphere* **59**: 831–835.

Sanghi, R., A. Dixit, and S. Guha (2006) Sequential batch culture studies for the decolorization of reactive dye by *Coriolus versicolor. Bioresour. Technol.* **97**: 396–400.

Schliephake, K., G.T. Lonergan, C.L. Jones, and D.E. Mainwaring (1993) Decolorization of a pigment plant effluent by *Pycnosporus cinnabarinus* in a packed-bed bioreactor. *Biotechnol. Lett.* **15**: 1185–1188.

Schliephake, K., D.E. Mainwaring, G.T. Lonergan, C.L. Jones, and W.L. Baker (2000) Transformation and degradation of the disazo dye Chicago Sky Blue by a purified laccase from *Pycnosporus cinnabarinus. Enzyme Microb. Technol.* **27**: 100–107.

Schneider, P., M.B. Caspersen, K. Mondorf, T. Halkier, et al. (1999) Characterization of a *Coprinus cinereus* laccase. *Enzyme Microb. Technol.* **25**: 502–508.

Schwitzguebel, J.-P., and T. Vanek (2003) Some fundamental advances for xenobiotic chemicals. In: *Phytoremediation: Transformation and Control of Contaminants*, S.C. McCutcheon and J.L. Schnoor, eds. Wiley-Interscience, Hoboken, NJ, pp. 123–157.

Selvam, K., K. Swaminathan, and K.-S. Chae (2003) Decolourization of azo dyes and a dye industry effluent by a white rot fungus *Thelephora* sp. *Bioresour. Technol.* **88**: 115–119.

Seshadri, S., P.L. Bishop, and A.M. Agha (1994) Anaerobic/aerobic treatment of selected azo dyes in wastewater. *Waste Manag.* **14**: 127–137.

Shaul, G.M., T.J. Holdsworth, C.R. Dempsey, and K.A. Dostal (1991) Fate of water soluble azo dyes in the activated sludge process. *Chemosphere* **22**: 107–119.

Shaw, T. (1998) The European Union integrated pollution prevention and control directive and its impact on the wool textile industry. *J. Soc. Dyers Colourists* **114**: 241–246.

Shen, H.-P., D.-G. Mou, K.-K. Lim, P. Feng, and C.-H. Chen (1992) Microbial decolorization of wastewater. U.S. patent 5,091,089.

Shin, K.-S., and C.-J. Kim (1998) Decolorization of artificial dyes by peroxidase from the white-rot fungus, *Pleurotus ostreatus*. *Biotechnol. Lett.* **20**: 569–572.

Shin, K.-S., I.K. Oh, and C.-J. Kim (1997) Production and purification of Remazol Brilliant Blue R decolorization peroxidase from the culture filtrate of *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* **63**: 1744–1748.

Shrivastava, R., V. Christian, and B.R.M. Vyas (2005) Enzymatic decolorization of sulfonphthalein dyes. *Enzyme Microb. Technol.* **36**: 333–337.

Soares, G.M.B., M.T. Pessoa de Amorim, and M. Costa-Ferreira (2001a) Use of laccase together with redox mediators to decolourize Remazol Brilliant Blue R. *J. Biotechnol.* **89**: 123–129.

Soares, G.M.B., M. Costa-Ferreira, and M.T. Pessoa de Amorim (2001b) Decolorization of an anthraquinone-type dye using a laccase formulation. *Bioresour. Technol.* **79**: 171–177.

Society of Dyers and Colourists (1976) *Color Index*, 3rd ed. Society of Dyers and Colourists, Yorkshire, UK.

Sosath, F., and J.A. Libra (1997) Biologische Behandlung von synthetischen Abwässern mit Azofarbstoffen. *Acta Hydrochim. Hydrobiol.* **25**: 259–264.

Spadaro, J.T., and V. Renganathan (1994) Peroxidase-catalyzed oxidation of azo dyes: mechanism of Disperse Yellow 3 degradation. *Arch. Biochem. Biophys.* **312**: 301–307.

Spadaro, J.T., M.H. Gold, and V. Renganathan (1992) Degradation of azo dyes by the lignin-degrading fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **58**: 2397–2401.

Srivastava, P.N., and A. Prakash (1991) Bio-accumulation of heavy metals by algae and wheat plants fed by textile effluents. *J. Ind. Pollut. Control Fed.* **7**: 25–30.

Stolz, A. (2001) Basic and applied aspects in the microbial degradation of azo dyes. *Appl. Microbiol. Biotechnol.* **56**: 69–80.

Sumathi, S., and B.S. Manju (2000) Uptake of reactive textile dyes by *Aspergillus foetidus*. *Enzyme Microb. Technol.* **27**: 347–355.

Sumathi, S., and V. Phatak (1999) Fungal treatment of bagasse based pulp and paper mill wastes. *Environ. Technol.* **19**: 93–98.

Swamy, J., and J.A. Ramsay (1999a) The evaluation of white-rot fungi in the decolorization of textile dyes. *Enzyme Microb. Technol.* **24**: 130–137.

Swamy, J., and J.A. Ramsay (1999b) Effects of glucose and NH_4^+ concentrations on sequential dye decolorization by *Trametes versicolor*. *Enzyme Microb. Technol.* **24**: 130–137.

Tan, B.H., T.T. Teng, and A.K.M. Omar (2000) Removal of dyes and industrial dye wastes by magnesium chloride. *Water Res.* **34**: 597–601.

Tatarko, M., and J.A. Bumpus (1998) Biodegradation of congo red by *Phanerochaete chrysosporium*. *Water Res.* **32**: 1713–1717.

Tayal, A.K., L. Das, and I. Kaur (1999) Biodegradation of pentachlorophenol (PCP) by white rot fungal strains screened from local sources and its estimation by high-performance liquid chromatography. *Biomed. Chromatogr.* **13**: 220–224.

Tekere, M., A.Y. Mswaka, R. Zvauya, and J.S. Read (2001) Growth, dye degradation and ligninolytic activity studies on Zimbabwean white rot fungi. *Enzyme Microb. Technol.* **28**: 420–426.

Toh, Y.-C., J.J.L. Yen, J.-P. Obbard, and Y.P. Ting (2003) Decolourization of azo dyes by white-rot fungi (WRF) isolated from Singapore. *Enzyme Microb. Technol.* **33**: 569–575.

Tychanowicz, G.K., A. Zilly, C.G.M. de Souza, and R.M. Peralta (2004) Decolourization of industrial dyes by solid-state cultures of *Pleurotus pulmonarius*. *Process Biochem.* **39**: 855–859.

USEPA (1993) *Toxics Release Inventory*. Public Data Release. Office of Pollution Prevention and Toxics, U.S. Environmental Protection Agency, Washington, DC.

Uygur, A. (1997) An overview of oxidative and photooxidative decolorisation treatments of textile waste waters. *J. Soc. Dyers Colourists* **113**: 211–217.

Vasdev, K., R.C. Kuhad, and R.K. Saxena (1995) Decolorization of triphenylmethane dyes by the bird's nest fungus by *Cyathus bulleri*. *Curr. Microbiol.* **30**: 269–272.

Vinodgopal, K., J. Peller, O. Makogon, and V. Kamat (1998) Ultrasonic mineralization of a reactive textile azo dye, Remazol Black B. *Water Res.* **32**: 3646–3650.

Wang, Y., and J. Yu (1998) Adsorption and degradation of synthetic dyes on the mycelium of *Trametes versicolor*. *Water Sci. Technol.* **38**: 233–238.

Willmott, N., J. Guthrie, and G. Nelson (1998) The biotechnology approach to colour removal from textile effluent. *J. Soc. Dyes Colourists* **114**: 38–41.

Wong, Y., and J. Yu (1999) Laccase-catalyzed decolorization of synthetic dyes. *Water Res.* **33**: 3512–3520.

Wu, F., H. Ozaki, Y. Terashima, T. Imada, and Y. Ohkouchi (1996) Activities of ligninolytic enzymes of the white rot fungus, *Phanerochaete chrysosporium* and its recalcitrant substance degradability. *Water Sci. Technol.* **34**: 69–78.

Wunch, K.G., T. Feibelman, and J.W. Bennett (1997) Screening of fungi capable of removing benzo[a]pyrene in culture. *Appl. Microbiol. Biotechnol.* **47**: 620–624.

Yang, F.-C., and J.-T. Yu (1996a) Development of a bioreactor system using immobilized white rot fungus for decolorization. I. Cell immobilization and repeated-batch decolourization tests. *Bioprocess Eng.* **15**: 307–310.

Yang, F.-C., and J.-T. Yu (1996b) Development of a bioreactor system using an immobilized white rot fungus for decolorization. II. Continuous decolourization tests. *Bioprocess Eng.* **16**: 9–11.

Yang, Q., A. Yediler, M. Yang, and A. Kettrup (2005) Decolorization of an azo dye, Reactive Black 5 and MnP production by yeast isolate: *Debaryomyces polymorphus*. *Biochem. Eng. J.* **24**: 249–253.

Yesilada, O. (1995) Decolourization of Crystal Violet by fungi. *World J. Microbiol. Biotechnol.* **11**: 601–602.

Yesilada, O., S. Cing, and D. Asma (2002) Decolourisation of the textile dye Astrazon Red FBL by *Funalia trogii* pellets. *Bioresour. Technol.* **81**: 155–157.

Yesilada, O., D. Asma, and S. Cing (2003) Decolorization of the textile dyes by fungal pellets. *Process Biochem.* **38**: 933–938.

Young, L., and J. Yu (1997) Ligninase-catalyzed decolorization of synthetic dyes. *Water Res.* **31**: 1187–1193.

Yu, Z., and X. Wen (2005) Screening and identification of yeasts for decolorizing synthetic dyes in industrial wastewater. *Int. Biodeterior. Biodegrad*. **56**: 109–114.

Zhang, F.-M., J.S. Knapp, and K.N. Tapley (1999) Development of bioreactor systems for decolorization of Orange II using white rot fungus. *Enzyme Microb. Technol.* **24**: 48–53.

Zheng, Z., R.E. Levin, J.L. Pinkham, and K. Shetty (1999) Decolorization of polymeric dyes by a novel *Penicillium* isolate. *Process Biochem.* **34**: 31–37.

Zhou, W., and W. Zimmermann (1993) Decolorization of industrial effluents containing reactive dyes by actinomycetes. *FEMS Microbiol. Lett.* **107**: 157–162.

Zollinger, H. (1991) *Color Chemistry*, 2nd ed. VCH, Weinheim, Germany.

11

FUNGAL BIOSORPTION OF HEAVY METALS

11.1 INTRODUCTION

The subtle and intricate process of removal of metals from aqueous solution has been known to the world for more than 5000 years, since Moses sweetened water using wood (Eccles, 1995). But microorganisms have been used to treat waste liquids since the end of the nineteenth century. However, the removal and/or recovery of metals from liquids or streams has received most attention during the past three decades.

Global industrialization is of great concern as a result of the release to the environment of toxic and persistent heavy metals that cause deleterious ecological effects and pose a serious threat to animals and humankind. The releases also lead to the mobilization of metals through leaching. Several industries—electroplating, electronic circuit production, steel and nonferrous processes, chemical and pharmaceutical, and others—discharge a variety of metal-laden wastewaters into the environment. Coal-fired power plants also produce large quantities of metal releases at several points during combustion. Release of industrial wastewater due to acid mine drainage over a large area is another big problem. In the United States, 389 of 703 National Priority List sites contain toxic metal contaminants and at least 100000 such sites are estimated in the European Union (Schmitt and Stitcher, 1991; Wilmoth et al., 1991). Government agencies have developed metal discharge standards in a number of countries to regulate such releases and contaminants in wastewaters entering waterways and sewerage systems.

Microorganisms have the ability to bind metals from aqueous solution. This phenomenon is known as *biosorption*, and the microorganisms responsible for the process are considered *biosorbents*. A wide variety of living and dead biomass of bacteria, algae, fungi, and plants is capable of sequestering toxic metals from waste streams. This is the foundation of *biosorption technology*, which offers a promising and economical alternative for the treatment of discharges of a wide variety of metal-containing industrial effluents. A plethora of literature exists on the biosorption of metal ions by bacteria, algae, fungi, and plants. Conservative estimates of new biosorbents in the North America environmental market amount to $27 million per year (Volesky, 2001). Yeasts and fungi are unique in metal biosorption, and this process is known as *mycosorption*. The fungal biomass used in mycosorption is termed *mycosorbent*. Mycosorption is a topic of great interest for researchers all over the world (Paknikar et al., 1998; Tobin, 2001; Malik, 2004).

11.2   BIOSORPTION AND BIOACCUMULATION OF HEAVY METALS

The process of biosorption and bioaccumulation of metals by microorganisms is not new. The accumulation of metals by fungi has received more attention in recent years because of its applications in environmental protection and recovery of metals. The biological removal of metals from solutions can be divided into three categories: (1) biosorption of metal ions on the surface of fungi, (2) intracellular uptake of metal ions, and (3) chemical transformation of metal ions by fungi. Living fungal biomass is required in the last two categories. Nonliving fungal biomass does not depend on requirements for growth, metabolic energy, and transport. In addition, nonliving biomass shows a strong affinity for metal ions due to the lack of protons produced during metabolism. The problem of toxicity of metals does not affect this type of biomass, which is seen as one of the major advantages of biosorption. Fungal biomass can be generated as a waste by-product of large-scale industrial fermentation and is pretreated by washing with acids and/or bases before final drying and granulation. All these factors contribute to reducing the final cost of the process.

Biosorption is a pseudo-ion-exchange process in which metal ion is exchanged for a counterion in the biomass or resin. In general, the filamentous fungi possess higher adsorption capacities for heavy metal removal. Aquatic fungi are also known to accumulate heavy metals. Uptake of metals was described by Michelot et al. (1998) and a tentative approach related to mechanisms of bioaccumulation in mushrooms was projected. The marine fungi *Corollospora lacera* and *Monodictys pelagica* have been found to accumulate lead and cadmium extracellularly in mycelia (Taboski et al., 2005). Biosorption involves a number of external factors (e.g., type of metal, ionic form in solution, and the functional site) and tends to be exothermic. Other factors,

such as pH, temperature, biomass concentration, type of biomass preparation, initial metal ion concentration and metal characteristics, and concentration of other interfering ions, are also important in evaluating the extent of biosorption. Biosorption and recovery can be intensified in the presence of stirring induced by magnetic field (Gorobets et al., 2004).

11.3   EVALUATION OF SORPTION PERFORMANCE

A great deal of confusion exists as to the evaluation of experimental data, despite the simple process of biosorption. This is due to the various methodologies and criteria used in the process. Another problem is evaluation of sorption performance when more than one metal is present in a system. At present, all theories and models are focused on single-metal systems. Due to limited knowledge on multimetal systems, it is difficult to reach predictive conclusions.

A plethora of literature exists on the evaluation of sorption performance. Adsorption is a well-known equilibrium separation process, and equilibrium data, known as *adsorption isotherms*, are basic requirements for design of the systems. The Langmuir and Freundlich equations are commonly employed in evaluating the adsorption isotherms at constant temperature. The Langmuir model is applicable to monolayer sorption onto a surface with a finite number of identical sites. The well-known *Langmuir model* is given by:

$$q = q_{\max} \frac{bC_f}{1 + bC_f}$$

where q (mg/g) is the uptake of the metal, $q_{\max}$ is the maximum uptake, C_f (mg/g) is the equilibrium (final) concentration of metal in the solution, and b is a constant.

The empirical *Freundlich model* based on sorption on a heterogeneous surface is given by:

$$q = KC_f^{1/n}$$

where K and n, the Freundlich constants, are indicators of adsorption capacity and adsorption intensity, respectively. The equation can be linearized in logarithmic form and the Freundlich constants can be determined.

Other biosorption isotherms are also known from the literature. These isotherms may not represent the physicochemical principles of the biosorption process and are not well understood. The models can determine the q versus C_f relationship as experimentally observed but do not provide any clues to the biosorption mechanism. The equilibrium models include the Scatchard–Langmuir, Redlich–Peterson, Brunauer–Emmett–Teller (BET), Radke–Prausnitz, and Dubinin–Radushkevich (DR). Some of these can be determined by the following equations:

Scatchard–Langmuir:

$$\frac{q}{C_f} = b\,q_{max} - b\,q$$

Brunauer–Emmett–Teller:

$$q = \frac{BQC_f}{(C_s - C)[1 + (B-1)(C_f / C_s)]}$$

where C_s is the saturation constant of the solute, B is a constant related to energy of interaction with the surface, and Q is the moles of solute adsorbed per unit weight of adsorbent in forming a complete monolayer on the surface.

Dubinin–Radushkevich:

$$\mathrm{In}q = \mathrm{In}qm - BE^2$$

where B is a constant related to the sorption energy and E is the Polanyi potential: $E = RT \ln(1 + 1/C_f)$.

These biosorption isotherms do not always provide a meaningful interpretation of data and are not applicable for systems operating under variable environmental conditions. This process generates irregular biosorption isotherms, due to variations related to multiple sites, the nature of the sorbent material, pH, and the complex chemistry of metal ions. Rapid uptake of metal by a fungal biosorbent is desirable. Two different sorption systems must be compared at the same equilibrium concentrations to obtain accurate results. The presence of other ions or co-cations also influences the sorption system by unnecessary interactions with the metal species.

11.4   MECHANISMS OF FUNGAL BIOSORPTION OF HEAVY METALS

Biosorption consists of several mechanisms that differ according to the fungal species used, the origin of the biomass, and its processing. These mechanisms include ion exchange, chelation, adsorption, crystallization, and precipitation, followed by ion entrapment in inter- and intrafibrillar capillaries, spaces of the polysaccharide material, and diffusion through the cell wall and membranes of fungi. Cell walls of fungi are composed of chitins, chitosans, and glucans and also contain proteins, lipids, and other polysaccharides. Yeast cell walls consist mainly of glucans and an outer layer of mannoprotein. Precipitation can occur in the cell wall components. The biomass usually contains a larger number and variety of functional groups or sites than those in monofunctional group ion-exchange resins. These sites include carboxyl,

sulfate, phosphate, hydroxyl, amino, imino, sulfonate, imidazole, sulfydryl, carbonyl, thioether, and other moieties. Certain fungal species are more effective and selective than others in removing particular metal ions from solution.

The mechanisms of fungal biosorption can be divided into two categories: metabolism independent and metabolism dependent. The first category employs live or dead biomass, and the second category transforms the metal internally coupled with the production of extracellular metabolites. The mechanisms of fungal biosorption are a topic of great interest to many authors; however, significant differences are recognized in the biosorbent mechanisms of fungal biomass. Electron microscopy, x-ray energy diffraction analysis, and infrared (IR) spectroscopy are also used to study binding mechanisms. The amino group of the cell walls of *Rhizopus nigricans* is involved in Cr(VI) binding from solution and from wastewater (Bai and Abraham, 2002). Chemical modification increases the number of active binding sites on the surface area that enhances the chromium adsorption capacity. The amine functional groups of *Mucor* cell walls also contributes to the removal of chromium from tanning effluent (Tobin and Roux, 1998). Fourier transform infrared (FTIR) spectroscopic analysis reveals the involvement of —COOH groups of acetone-washed yeast biomass in lead biosorption (Ashkenazy et al., 1997). The —COOH groups also contribute to the binding sites of metals in the cell walls of *Mucor rouxii* (Gardea-Torresdey et al., 1996). Electron microscopy, x-ray energy diffraction analysis, and IR spectroscopy reveal three hypotheses of the mechanism of lead uptake (Zhang et al., 1998). NaOH-treated and NaOH-untreated biomass establish that biosorption occurs in the chitin structure of cell walls. Electron microscopy reveals the localization of nickel on the cell surface of *Rhizopus* sp. 0101 (Mogollon et al., 1998). Transmission electron microscopy (TEM) shows that Pb(II) is associated in the cell wall and membrane after 3 minutes and cytoplasm after 2 hours in *Saccharomyces cerevisiae* (Suh et al., 1998). A three-step mechanism of Pb(II) accumulation is advocated. The first step is metabolism independent, the second step is metabolism dependent, and the third step is metabolism dependent or independent after 24 hours. Two mechanisms govern the removal of Cr(VI) from the aqueous solution by dead biomass of *Aspergillus niger* (Park et al., 2005). During mechanism I, Cr(VI) is reduced directly to Cr(III) by contact with the biomass. Mechanism II consists of three steps: the binding of Cr(VI) to positively charged groups in the cell wall, reduction of Cr(VI) to Cr(III) by adjacent functional groups, and release of Cr(III) by electron repulsion.

Fungi can remove both soluble and insoluble metals from solution and can leach metals from solid wastes. Fungi produce protons, organic acids, phosphatases, and other metabolites for solubilization. Many heterotrophic fungi produce organic acids that assist in solubility and complexing of metal cations. Several fungi are known to produce large amounts of different kinds of acids that assist in metal leaching purposes. Oxalic acid is a leaching agent for a

variety of metals, such as Al, Fe, and Li, forming soluble metal oxalate complexes. Metal oxalates are also produced by a wide range of fungi, including mycorrhizas and lichenicolous fungi. The white-rot fungi *Bjerkandera fumosa*, *Phlebia radiata*, and *Trametes versicolor* and the brown-rot fungus *Fomitopsis pinicola* produce oxalate crystals in high levels on ZnO, $Co_3(PO_4)_2$, and $CaCO_3$ (Jarosz-Wilkolazka and Gadd, 2003). In brown-rot fungi, induction of oxalic acid is related to copper tolerance (Green and Clausen, 2003). Brown-rot fungi can maintain oxalic acid concentrations as high as $600 \mu M/g$. Oxalic acid is also produced by brown-rot fungi during leaching of metals from the treated wood (Humar et al., 2004). One-third of the isolates of soil fungi are able to solubilize at least one toxic metal compound, ZnO, $Co_3(PO_4)_2$, and $Zn_3(PO_4)_2$, and 10% solubilize all three (Sayer et al., 1995). In *Penicillium simplicissimum*, adsorption of zinc is accompanied by the production of citric acid (Franz et al., 1991). The cultural filtrate of *Aspergillus niger* can render the solubility of 18% Cu, 7% Ni, and 4% Co, and these amounts are enhanced by the addition of HCl (Sukla et al., 1992). Fe(III) can be solubilized by a low-molecular-weight chelating compound known as the ferrichrome (Crichton, 1991). The structure of the hyphae in the form of hard compact pellets is altered due to a manganese deficiency (Schreferl et al., 1986). *Penicillium janthinellum* F-13 on different media reduces Al toxicity, but tolerance of the high external concentration of Al appears to be due to a different mechanism (Zhang et al., 2002).

The mechanisms of fungal transformation are reduction, methylation, and dealkylation of metals (Table 11.1). Certain species of *Penicillium* are also known to remove iron from alloys (Siegel et al., 1990). *Alternaria alternata* causes volatilization of substantial amounts of selenium to the dimethylselenide form (Thompson-Eagle et al., 1991). The volatilization process is optimized and used in the fungal bioremediation of contaminated water and land (Thompson-Eagle and Frankenberger, 1992). The mechanism of fungal selenium transformations has been described (Thompson-Eagle and Frankenberger, 1992; Gadd, 1993). Several fungal species (i.e., *Candida humicola*, *Gliocladium roseum*, and *Penicillium* sp.) have been established to methylate arsenic compounds such as arsenate [As(V), AsO_4^{3-}], arsenite [As(III), AsO_2^-], and methylarsonic acid [$CH_3H_2AsO_3$] to volatile dimethyl-[$(CH_3)_2HAs$] or trimethylarsine [$(CH_3)_3As$] (Tamaki and Frankenberger, 1992). *Fusarium oxysporum* reduces silver ions in solution, thus forming stable silver hydrosol (Ahmad et al., 2003). Siver nanoparticles of 5 to 15 nm are stabilized by proteins of the fungus. It seems that the reduction of silver ions occurs due to an enzymatic process. A $FeCl_3$-pretreated waste tea fungal mat is an effective biosorbent for As(III) and As(V), and an autoclaved fungal mat is effective for Fe(II) removal from a groundwater sample (Murugesan et al., 2006). Modification of mobility and toxicity of metalloids by these processes can lead to their biotechnological potential in bioremediation. For more discussion of the mechanisms of mycotransformation of metals, the reader is referred to a review by Gadd (2001).

TABLE 11.1 Fungal Transformation of Metals, Metalloids, and Organometals

Fungus	Transformation	Mechanism	Reference
Aureobasidium pullulans	$Ag(I) \rightarrow Ag(0)$	Reduction	Kierans et al., 1991
Candida albicans			
Debaryomyces hansenii			
Rhodotorula rubra			
Saccharomyces cerevisiae			
S. cerevisiae	$Fe(III) \rightarrow Fe(II)$	Reduction and nonreduction	Lesuisse and Labbe, 1989
Fusarium sp.	$Se(IV)$ or $Se(VI) \rightarrow Se(0)$	Reduction	Gharieb et al., 1995
Fusarium sp.	$Te(II) \rightarrow Te(0)$	Reduction	Gharieb et al., 1999
Penicillium citrinum			
Schizosaccharomyces pombe	$Te(IV)$ or $Te(VI) \rightarrow Te(0)$	Reduction	Smith, 1974
C. albicans	$Hg(II) \rightarrow Hg(0)$	Reduction	Yannai et al., 1991
S. cerevisiae			
Alternaria alternata	$Se(IV)$ or $Se(VI) \rightarrow (CH_3)_2Se$	Methylation	Thompson-Eagle et al., 1991
Penicillium sp.	$Te(IV)$ or $Te(VI) \rightarrow (CH_3)_2Te$	Methylation	Huysmans and Frankenberger, 1991
	$CH_3\ AsH_2O_3 \rightarrow (CH_3)_3As$		
Penicillium sp. MR-2	Organic $Hg \rightarrow Hg(II) \rightarrow Hg(0)$	Dealkylation	Tezuka and Takasaki, 1988

11.5 FUNGAL BIOSORPTION REACTORS FOR HEAVY METALS

11.5.1 Types of Reactors

In recent years, fungal biosorption reactors have been well developed (Table 11.2) but not commercialized. The process of biosorption is basically a solid–liquid contact and requires refinement in the light of variable conditions. Based on appropriate contact between the metal solution and biosorbent, several types of biosorption reactors have been recognized: batch stirred-tank, continuous-flow stirred-tank, fixed packed-bed, pulsating-bed, fluidized-bed, multiple-bed, and others.

11.5.1.1 Batch Stirred-Tank Reactors. Batch reactors were one of the earliest used for the effectiveness of metal–biosorbent systems. A plug-flow biosorption reactor with a limited retention time for the removal of copper is depicted in Figure 11.1 (Butter et al., 1998). The metal-containing influent and biomass suspension are pumped to an in-line mixer. The mixed solution flows in a pipe sufficient for a retention time of 2 to 5 minutes. Polyelectrolyte solution is added from the second in-line mixer, resulting in flocculation of biomass, and this solution is then discharged into the settling tank. This process is operated continuously at a flow rate of 1.5 l/m with a 87% reduction in copper. Batch stirred-tank reactors in series are also known for their adsorption of Cu(II) and Ni(II) by *Rhizopus arrhizus* (Sag and Kutsal, 1995).

11.5.1.2 Continuous-Flow Stirred-Tank Reactors. In a continuous-flow stirred-tank reactor (CFST), the chamber or vessel is similar to that used for batch systems; however, there is a continuous supply of the metal solution. Based on the operations, two basic types are known: (1) a type using a continuous feed of fresh biosorbent, which is then harvested continuously from the bioreactor in the effluent, and (2) a type that retains a batch of the biosorbent in the system by option in the reactor. At present, little is known about the biosorption of metals involving the CFST. However, heavy metals have been removed in a CFST using *Rhizopus arrhizus* (Sag et al., 2000b).

11.5.1.3 Fixed Packed-Bed Reactors. *Pycnosporus sanguineus* has the ability to adsorb heavy metals from aqueous solution using the fixed-bed column shown in Figure 11.2 (Zulfadhly et al., 2001). The biosorption process utilizes a Perspex column of 5 cm internal diameter, 0.2 cm wall thickness, and 1 m length. In general, the *P. sanguineus* biomass removes more than 90% of Pb(II), Cu(II), and Cd(II). The lead uptake increases with an increase in bed height from 4 cm to 15 cm. The efficiency of the process remains the same for the first two cycles, then drops a little in the third and fourth cycles.

11.5.1.4 Immobilized Reactors. Immobilized cell systems are known to be exploited in commercially oriented biosorption research. The advantages

TABLE 11.2 Fungal Bioreactors for Biosorption of Heavy Metals

Fungus/Biomass Concentration	Bioreactor Configuration/Volume	Flow Rate	Metal/Conc.	Metal Uptake Capacity/Removal (%)	Eluant/Reuse	Duration (minutes/hours/days)	Reference
Penicillium chrysogenum (4 g/l)	Plug-flow continuous mode	1.5 l/m	Cu(II), 10.12 mg/l	87		2–5 m	Butter et al., 1998
Rhizopus arrhizus (1 g/l)	Continuous-flow stirred-tank contactor	3 ml/m	Pb(II), 58 mg/l; Ni(II), 51 mg/l; Cu(II), 51 mg/l	36; 16; 13		240 m	Sag et al., 2000b
Pycnosporus sanguineus	Fixed packed bed, bed height 15 cm	25 ml/m	Pb(II); Cu(II); Cd(II), 79–367 mg/l	>90; >90; >90	0.1 M HCl/ 4 cycles	250 m	Zulfadhly et al., 2001
Aspergillus oryzae (pellets, 2 g)	Chemostat, 1 L, bed height 36 cm	3.4 ml/m	Cu(II), 5×10^{-5} M	>99	5×10^{-3} M HClO$_4$	16 m	Huang and Huang, 1996
Aspergillus niger (4.7 g)	Immobilized in a polysulfone matrix, continuous column, bed height 24.5 cm	3 ml/m	Pb(II), 10 mg/l; Cd(II), 10 mg/l; Cu(II), 10 mg/l; Ni(II), 6 mg/l	>50; >50; >50; >50	0.05 N HNO$_3$		Kapoor and Viraraghavan, 1998b
Saccharomyces cerevisiae (2 g/dm^3)	Immobilized in PVA gel		Cu(II), 0.5 mM/dm^3	97	10 mM/dm^3 HCl/5 cycles	24 h	Ting and Sun, 2000
S. cerevisiae (pellets, 5–10 g)	PVA Na-*ortho*-PO$_4$; PVA Na-alginate; Alkali-treated PEI:GA		Cu(II), 100 mg/l	>1 mg/g; 5 mg/g; 10 mg/g	1 mM EDTA and Milli-Q water		Stoll and Duncan, 1997
Fomitopsis pinicola CCBAS 535	Free cells; Immobilized in alginate beads		Cd(II), 10 mg/l	40–48; 28–46		10 d	Lebeau et al., 2002

Organism	Immobilization	Flow rate	Metal, concentration	Uptake	Desorption	Time	Reference
Mucor rouxii (4.5 g)	Immobilized in a polysulfone matrix	2.22–2.66 ml/min	Pb(II), 10 mg/l; Cd(II), 10 mg/l; Ni(II), 10 mg/l; Zn(II), 10 mg/l	4.0 mg/g; 3.7 mg/g; 0.36 g/g; 1.36 mg/g	0.05N HNO_3 and water		Yan and Viraraghavan, 2001
R. arrhizus	Immobilized in alginate beads/ fluidized-bed stirred tank		Cr(VI), 100 mg/l	94		4h	Prakasham et al., 1999
R. arrhizus	Immobilized in Ca-alginate		^{241}Am, 1.08 MBq/l	85; 94	EDTA and 2M/l HNO_3	2h	Liao et al., 2004
S. cerevisiae	Immobilized in sepiolite, column reactor bed height 1.5 cm	3 ml/m	Cr(III); Cr(VI), 0.1 μg/ml	96; 97	1M/l HCl/ 20 cycles		Bag et al., 2000
Lentinus sajor-caju (25 mg)	Live mycelia; Dead mycelia; Immobilized in alginate gel		Cd(II), 400 mg/l	78; 90	10mM HCl/ 3 cycles	60m	Bayramoglu et al., 2002
Trametes versicolor	Live mycelia; Heat-killed mycelia; Immobilized on carboxymethyl cellulose		Cd(II), 30–700 mg/l	124 mg/g; 153 mg/g	10mM HCl/ 5 cycles	60m	Yalcinkaya et al., 2002
T. versicolor (25 mg)	Live mycelia; Dead mycelia; Immobilized in Ca–alginate beads		Cd(II), 600 mg/l	102 mg/g; 120 mg/g	10mM HCl/ 3 cycles	60m	Arica et al., 2001
T. versicolor (1 g)	Live mycelia; Heat-inactivated mycelia; Immobilized onto CMC beads		Cu(II), 200 mg/l; Pb(II), 200 mg/l; Zn(II), 200 mg/l; Cu(II), 200 mg/l; Pb(II), 200 mg/l; Zn(II), 200 mg/l	1.51 mM; 0.85 mM; 1.33 mM; 1.84 mM; 1.11 mM; 1.67 mM	10mM HCl/ 5 cycles	60m	Bayramoglu et al., 2003

TABLE 11.2 Continued

Fungus/Biomass Concentration	Bioreactor Configuration/ Volume	Flow Rate	Metal/Conc.	Metal Uptake Capacity/ Removal (%)	Eluant/ Reuse	Duration (minutes/ hours/days)	Reference
Phanerochaete chrysosporium	Live mycelia Heat-inactivated mycelia Immobilized onto Ca–alginate beads		Hg(II), 500 mg/l Cd(II), 500 mg/l Hg(II), 500 mg/l Cd(II), 500 mg/l	66 mg/g 50 mg/g 112 mg/g 85 mg/g	10 mM HCl/ 3 cycles	60 m	Kacar et al., 2002
Funalia trogii	Live mycelia Heat-inactivated mycelia Immobilized onto Ca–alginate gel beads		Hg(II), 200 mg/l Cd(II), 200 mg/l Zn(II), 200 mg/l Hg(II), 200 mg/l Cd(II), 200 mg/l Zn(II), 200 mg/l	333.0 mg/g 164.8 mg/g 42.1 mg/g 403.2 mg/g 191.6 mg/g 54.0 mg/g	10 mM HCl/ 5 cycles	60 m	Arica et al., 2004
Aspergillus terreus	Immobilized in polyurethane foam		Fe Cr Ni(II) effluent	164 mg/g 96 mg/g 20 mg/g		6 d	Dias et al., 2002
T. versicolor *P. chrysosporium*	Immobilized in carboxymethyl cellulose		$UO_2(II)$, 100–1000 mg/l synthetic wastewater	309.1 mg/g 158 mg/g	10 mM HCl/ 5 cycles	20 m	Genc et al., 2003
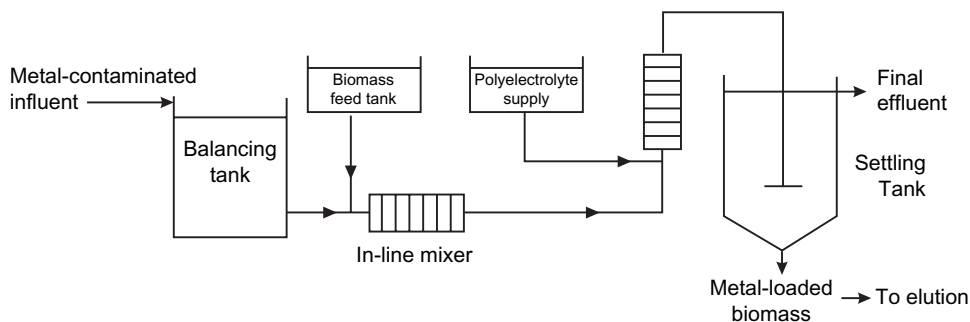
Figure 11.1 Plug-flow biosorption by *Penicillium chrysogenum*. [Reprinted from Butter et al. (1998), copyright © with permission from IWA.]
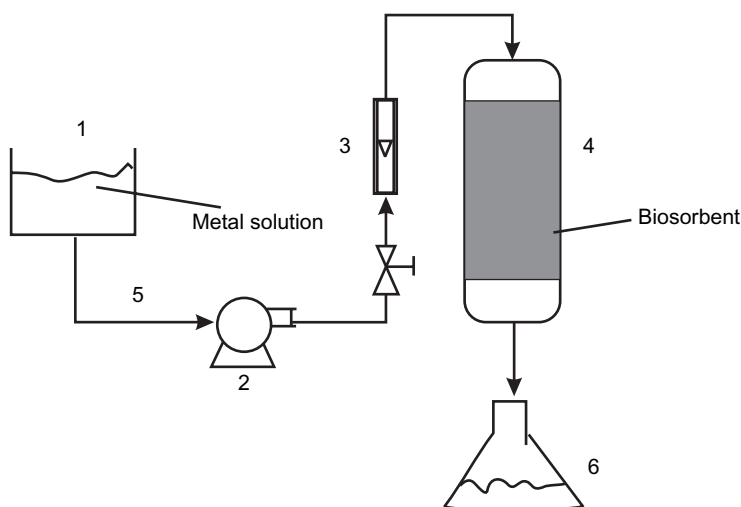
Figure 11.2 Fixed-bed column for biosorption of metals by *Pycnosporus sanguineus*. 1, Feed storage; 2, pump; 3, rotameter; 4, column; 5, delivery line; 6, sample collection. [Reprinted from Zulfadhly et al. (2001), copyright © with permission from Elsevier.]

of immobilized cell systems lie in being easily modeled, in reusing biomass, and in phase separation after metal uptake. Dead fungal biomass can be immobilized in polyacrylamide, alginate, polysulfone, textile fibers, and inorganic compounds for the removal of metals. Biomass can be immobilized by three techniques: entrapment, encapsulation, and bonding. Many filamentous fungi tend to form pellets in submerged cultures. So it is necessary to examine the physiological characteristics of the mycelium and the physicochemical factors in relation to pellet formation. Immobilized fungal cells increase the

size of the individual biosorbent particles. Increased removal efficiencies and reduced pressure drops coupled with low capital and low operation and space costs make this process attractive. Also, high flow rates can be handled in immobilized reactors with less chance of clogging. Immobilized biomass can be used in stirred-tank and packed-bed systems. Variants of packed-bed systems include stirred-tank and airlift fixed-bed or fluidized-bed bioreactors.

Biomass–polysulfone beads for immobilizing *Aspergillus niger* display good handling and settling qualities and the biomass loss is negligible during the process (Kapoor and Viraraghavan, 1998b). A polyvinyl alcohol (PVA) matrix for inactivated *Saccharomyces cerevisiae* for copper biosorption displays a low mass-transfer resistance (Ting and Sun, 2000). Immobilization of nonviable *S. cerevisiae* in PVA Na–alginate and alkali-treated polyethylenimine:glutaraldehyde (PEI:GA) shows the highest Cu(II)-accumulating capacity (80%), which can be increased by regeneration and reuse of the pellets (Stoll and Duncan, 1997). The Thomas model has been found suitable for the column kinetics of metal removal using immobilized *Mucor rouxii* biomass in polysulfone matrix (Yan and Viraraghavan, 2001). The biosorption of metals on the carboxymethyl cellulose and both immobilized live and heat-killed *Trametes versicolor* preparations increases with an increase in initial concentration of these ions (Yalcinkaya et al., 2002; Bayramoglu et al., 2003). An immobilized biomass of *Fomitopsis carnea* on PVA beads showed 80% gold removal as compared to that on immobilized calcium–alginate beads (Khoo and Ting, 2001). Dead fungal biomass immobilized in polyacrylonitrile-B/75% removed metals from simulated wastewaters (Zouboulis et al., 2003). The bioreactor shown in Figure 11.3 was developed for the detoxification of Cr(VI) in contaminated soils (Krishna and Philip, 2005). More than 80% Cr(VI) reduction has been obtained at an initial Cr(VI) concentration of 50 mg/l within 4 hours. The adsorption column of powdered *Ganoderma lucidum* was used as a polishing treatment unit, and this removed more than 95% of Cr(III) within 3 hours. This biosystem is a feasible option for the ex situ treatment of Cr(VI)-contaminated soils.

11.5.2   Models of Process Development

Mathematical models and computer simulations are important tools in the development of technologies from the laboratory to full-scale application at various levels. Models also assist in analyzing the experimental data and process designs, and their optimization is necessary in evaluating the response of systems under variable operating conditions. Computer simulations are generally a new area and can contribute to the realistic predictions of the process. For further information on the equilibrium and kinetic models, including the applications of fungal biosorption, the reader is referred to a recent review (Aksu, 2005). The different biosorption models and their applicability related to fungal systems are integrated in Table 11.3.
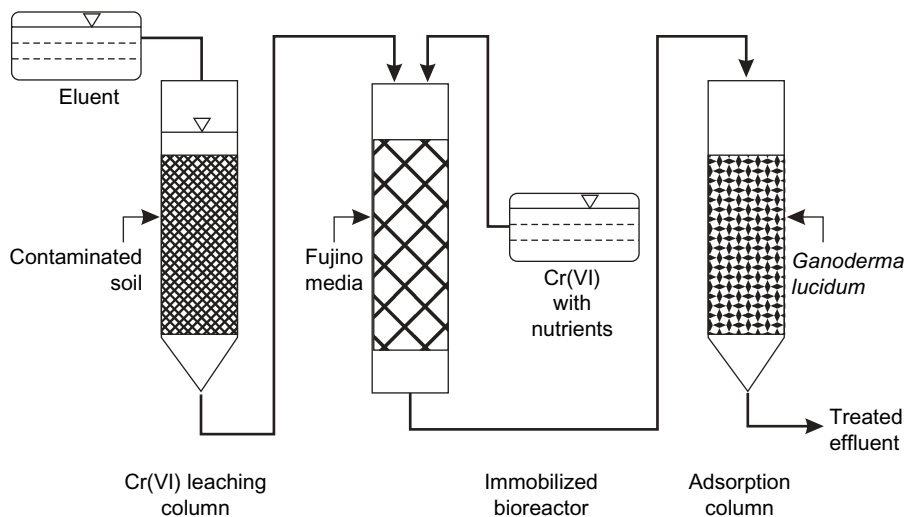
Figure 11.3 Bioremediation of Cr(VI)-contaminated soils using *Ganoderma lucidum*. [Reprinted from Krishna and Philip (2005), copyright © with permission from Elsevier.]

TABLE 11.3 Models for Development of Fungal Biosorption

Model	Applicability	Reference
Bohart–Adams sorption model	Analysis of biosorption-column performance; several limitations; no longer in use	Faust and Aly, 1987
Equilibrium column model	Assessment of multicomponent ion exchange processes in column; several limitations; uncertain future	Helfferich, 1967; Klein et al., 1967; Tondeur and Klein, 1967
Kinetic model	Calculation of solid-phase concentration of metals; adsorption capacity of bed	Thomas, 1948
Mass transfer model	Predict breakthrough curves for all species; simulated under different conditions	Tan and Spinner, 1994
Finite difference computer model	Simulation of performance of column reactors	Huang et al., 1989
Mathematical model	Performance of mono-metal and multicomponent biosorption	Sag et al., 2000b, 2001
Mathematical model	Prediction of mass transfer coefficient and diffusivity of metals	Brauch and Schlunder, 1975
Bed depth service time model	Design of biosorption columns	Mckay and Bino, 1990

11.5.3   Desorption and Regeneration

The objective of desorption is to regenerate the biomass for the next step in biosorption and recovery of biosorbed metal. Selection of the desorbent and the desorption process are important so that these can easily integrate into the entire process of fungal biosorption. The regeneration of the biosorbent reduces the cost of the biosorption process and opens the door for the recovery of metal from the liquid phase. Several articles have been published on varying treatments with little or no loss in biosorptive capacity. Metal elution, elution potential and efficiencies, and recovery from the biomass have been discussed (Singleton and Tobin, 1996). Common as well as effective metal eluants used for the regeneration of fungal biomass are listed in Table 11.4.

Sodium bicarbonate proves a good eluter for some fungi belonging to Mucorales (Galun et al., 1983). Treatment of *Rhizopus nigricans* biomass with 0.1 N NaOH and formaldehyde resulted in deterioration of the adsorption capacity (Bai and Abraham, 2002). Extraction with alcohol, acetone, and HCl improved the uptake capacity. Boiling of *Saccharomyces uvarum* biomass enhanced the adsorption of Pb (Ashkenazy et al., 1997). More than 90% of the biosorbed metal, equivalent to a 25-fold increase in copper concentration, was eluted (Butter et al., 1998). Copper was recovered from the eluant by electrolysis using a rotating cathode cell. Treating *Saccharomyces cerevisiae* with glucose increases the energy supply to yeast cells to accumulate more metals from solution (Mapolelo and Torto, 2004).

TABLE 11.4   Common Metal Eluants for Regeneration of Fungal Biomass

Mechanism	Common Metal Eluants	Concentration Range	Time (hours/ minutes)
Counterions	Salts of sodium, potassium, and calcium (chlorides, carbonates, bicarbonates, and hydroxides)	0.1–1 N	1–2 h
Proton exchangers	Mineral acids, such as HCl, H_2SO_4, $HClO_4$, and HNO_3	0.01–1 N	1–2 h
Complexing agents	Ethylenediaminetetraacetic acid (EDTA), nitriloacetic acid (NTAA)	0.01–0.1 N	2 h
Heat treatment	Boiling		5 m
Combinations	Basic treatment followed by acidic treatment, and vice versa		

11.5.4   Effect of Effluent Composition

In general, effluents contain more than one metal species. In biosorption experiments, a great competition exists for a limited number of binding sites for several metal species. In column experiments, affinities and concentration of metals play an important role in the success of the efficiency of the biosorption. Due to competition, some metals in trace or smaller amounts may reach a breakthrough curve before others in the column. The role of affinity of various metals and the criteria to reach breakthrough related to a specific metal in multimetal solutions or effluents has not yet been explained.

11.6   APPLICATIONS OF FUNGAL BIOSORPTION OF HEAVY METALS

11.6.1   Biosorption by Filamentous Fungi

Different types of biomass have been tested for their metal-binding capacities under certain conditions. Selection of biomass depends on the sequestering ability of the metal ions. Some types of sorbent has a broad range with no specificity; others are specific for certain metals. Fungal biosorbents have been described by several researchers. The characteristics of the fungal biomass for suitability in the process are (1) biosorption efficiency and metal uptake capacity and affinity for metal ions, (2) specificity for metal ions, (3) flocculation and sedimentation efficiency, (4) desorption efficiency, and (5) preservation of the biosorptive capacity. Fungal melanins of filamentous fungi also contribute to the removal of metals, and their interactions with metals have been explained by Fogarty and Tobin (1996).

 The first step in biosorption is the choice of metals. Uranium is given the highest consideration, due to great interest by the nuclear industry. Mercury, lead, and cadmium are next, due to strong toxic environmental effects. Chromium is highly toxic and must be reduced to a trivalent state that is more amenable to removal. Copper is used in many applications and is also increasing in the environment. Little is known as to the removal of radium, thorium, strontium, and neodymium by filamentous fungi. Table 11.5 lists the uptake of heavy metals by filamentous fungi. Fungi belonging to the Order Mucorales are good biosorbents of uranium (Volesky, 1994). Metal enrichment selectivity by *Mucor hiemalis* is in the decreasing order $Cr(III) > Cu(II) > Pb(II) > Ag(I) > Al(III) > Co(II) > Zn(II) > Ni(II) > Fe(II) > Mo(V) > Cd(II) > Cs(I) > Cr_2O_7^{2-} > CrO_4^{2-} > VO^{3-}$ (Pillichshammer et al., 1995). The dead biomass of *Rhizopus nigricans* has been shown to be a potent biosorbent of Cr(VI) ions (Bai and Abraham, 2002). *Rhizopus* sp. 0101 biosorbed 80% of Ni(II) within 2 hours at an initial concentration of 10 ppm (Mogollon et al., 1998). *Aspergillus niger* removed 0.52 mg Cu(II)/g dry weight and *A. pendulus* 0.09 mg Cu(II)/g dry weight (Price et al., 2001). Electron microscopy has

TABLE 11.5 Uptake of Heavy Metals by Filamentous Fungi

Fungus/Biomass Concentration	Metal/Conc.	Metal Uptake Capacity/ Removal (%)	pH (units)	Eluant/Reuse	Incubation (minutes/ hours/days)	Reference
Aspergillus flavus (1 g dry weight)	U(VI), 1×10^{-3} M	40 mg/g	4.5	0.1N HCl	2h	Hafez et al., 1997
Mucor hiemalis	Th(IV), 2.5×10^{-4} M	8 mg/g	2.5			
M. javanicus	UO$_2$(II), 125 mg/l	229 mg/g	5.8		1h	Tsuruta, 2002
Neurospora sitophila		343 mg/g				
Penicillium chrysogenum		285 mg/g				
P. lilacinum		101 mg/g				
Rhizopus arrhizus		154 mg/g				
Rhizopus oryzae		170 mg/g				
Trichoderma viride (15 mg dry weight)		227 mg/g				
		98 mg/g				
Phellinus badius	Pb(II)	170 mg/g	5.0		15 m	Matheickal and Yu, 1997
P. chrysogenum	Pb(II)	116 mg/g	4.0–5.0			Niu et al., 1993
	Cd(II)	11 mg/g				
	Cu(II)	9 mg/g				
Rhizopus nigricans (2 g)	Pb(II), 500 mg/l	68 mg/g	4.0	0.5M HCl, HNO$_3$, 0.2M EDTA	120 m	Zhang et al., 1998
Aspergillus niger	Pb(II), 200 mg/l	580 mg/g	5.0		20 m	Lo et al., 1999
Mucor rouxii (0.05–1 g/l)		74 mg/g	6.0			
M. rouxii, 0.05 g Live biomass	Pb(II), 10 mg/l	35.69 mg/g	5.0	0.05M HNO$_3$, 0.2M CaCl$_2$, or 0.2M NaCl	10–14h	Yan and Viraraghavan, 2003
	Ni(II), 10 mg/l	11.09 mg/g				
	Cd(II), 10 mg/l	8.46 mg/g				
	Zn(II), 10 mg/l	7.75 mg/g				

Biomass/Organism	Metal, concentration	Capacity	pH	Treatment	Time	Reference
Dead biomass	Pb(II), 10mg/l Ni(II), 10mg/l Cd(II), 10mg/l Zn(II), 10mg/l	53.75mg/g 20.49mg/g 20.31mg/g 53.85mg/g	6.0			Tobin and Roux, 1998
Mucor miehi	Cr(III), 1.77g/l	1.15mM/g 0.7mM/g	4.0 2.0	1N NaOH, 1N H_2SO_4, and vice versa	8h	
R. nigricans (0.2%) APTS-treated biomass	Cr(VI), 500mg/l	212mg/g	2.0			Bai and Abraham, 2002
PEI-treated biomass		160mg/g				
R. arrhizus (1 g/l)	Fe(III), 100–125mg/l Cr(VI), 125–150mg/l Ni(II), 125–200mg/l	3.9mg/g per minute 8.4mg/g per minute 10.3mg/g per minute	2.0		30m	Sag and Kutsal, 2000
R. oryzae	Cd(II), 0.2–1.2mM	0.28mM/g	6.0	Calcium solution and heat	24h	Yin et al., 1999
Rhizopus oligosporus		0.35mM/g				
Aspergillus oryzae		0.40mM/g				
R. arrhizus		0.56mM/g				
R. arrhizus	Pb(II)	0.61mM/g				
R. arrhizus	Cu(II)	0.60mM/g				
R. arrhizus (2 g dry weight/l)	Zn(II), 0.2–1.2mM	0.53mM/g				
Aspergillus terreus *Cladosporium cladosporioides*	Cd(II), 10mg/l	70 63	6.0		13d	Massaccesi et al, 2002

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TABLE 11.5 *Continued*

Fungus/Biomass Concentration	Metal/Conc.	Metal Uptake Capacity/Removal (%)	pH (units)	Eluant/Reuse	Incubation (minutes/hours/days)	Reference
Fusarium oxysporum		63				
Gliocladium roseum		65				
Penicillium sp.		60				
Trichoderma koningii		64				
A. oryzae	Cu(II), 5×10^{-5}	55.57 µM/g	5.0			Huang and Huang, 1996
R. oryzae (1g/l)						Zhou, 1999
R. arrhizus (1 g/l)	Zn(II), 20–140 µM/l	213 µM/g^{-1}	5.5			
Neurospora crassa	Co(II), 500 mg/l	90			24h	Karna et al., 1996
N. crassa						
Mutant *CSM-1*	^{60}Co, Fe(II)	295.3 ng/g, 17.3 mg/g	5.1		44h	Rashmi et al., 2004
Mutant *CSM-11*	^{60}Co, Fe(II)	331 ng/g, 13.6 mg/g				
Penicillium citrinum	^{60}Co, Fe(II) 0.160 µM, 9.45 mM	126.4 ng/g, 13.6 mg/g				
Baker's yeast F	Neodymium, 8.71 g/l	314 mg/g 89 mg/g	1.5		2h	Palmieri et al., 2000a
Phomopsis sp. (0.1 g/l)						
Phomopsis sp. (biomaterial 20mg)	Pb(II), Cu(II), 1mM/l; Cd(II), Zn(II), 1mM/l; Ni(II), 1mM/l	870, 390 µM/g; 230, 130 µM/g; 110 µM/g	6.0	0.25 M HNO$_3$	24h	Saiano et al., 2005

revealed the presence of a strongly electron-dense layer of Th(IV) and U(VI) ions around the surface of cell walls of *Aspergillus flavus* after 2 hours (Hafez et al., 1997). Scanning electron microscopy coupled with x-ray energy dispersion analysis indicated that K(I) and Ca(II) are replaced by Pb(II) on the cell wall of *Mucor rouxii* (Lo et al., 1999). Chitin and chitosan of the cell wall are the predominant targets for zinc biosorption by *Rhizopus arrhizus* (Zhou, 1999). Lead uptake by nonliving *R. nigricans* was reduced in the presence of co-cations such as Zn(II) and Fe(III) ions at pH 4.0 (Zhang et al., 1998). *Trichoderma viride* was shown to survive the high metal concentration of Cu(II), Zn(II), and Cd(II) as a result of resistant cells (Errasquin and Vazquez, 2003). This high tolerance appears to be due to its isolation from sludge containing high levels of metals.

Uptake of zinc by fungi has been summarized by Ross (1994). Zn(II) uptake by the yeast–mycelium dimorphic fungus *Aureobasidium pullulans* grown in chemostat culture correlated with the saturation kinetics (Krogh et al., 1998). Zinc uptake affected the yeast–mycelium transition of *Candida albicans* (Sabie and Gadd, 1990). Strain 1 of *Cladosporium cladosporioides* sorbed gold and silver, and strain 2 sorbed copper and cadmium in addition to gold and silver (Pethkar et al., 2001). Strain 2 had 150 times the cell wall hexosamine content of strain 1. Hexosamine was responsible for nonspecific metal binding; other cell wall polymers played a role in preciousmetal binding. *Phoma* sp. selectively accumulated silver (Pighi et al., 1989). Metal ions in both the distilled water and urban wastewater were adsorbed in the order Pb(II) > Cu(II) > Zn(II) > Cd(II) > Ni(II) using biomaterials obtained by thermal alkali treatment of *Phomopsis* sp. (Saiano et al., 2005).

The ability of filamentous fungi to adsorb radionuclides from the solution is well recognized. Among different fungi, the mutant varieties (*CSM-I* and *CSM-II*) of *Neurospora crassa* have the highest ^{60}Co and Fe(II) pickup capacities under stationary conditions (Rashmi et al., 2004). However, *CSM-1* and *CSM-II* showed a 75% and 50% decrease in ^{60}Co pickup capacities under shaking conditions. *Penicillium citrinum* exhibited twice the increase in ^{60}Co pickup capacity under shaking conditions. *Rhizopus arrhizus* sequestered more than 99% of the total ^{241}Am from a solution of 5.6 to 111 MBq/l (44–877 μg/l) with adsorption capacities of 4 to 79 MBq/g biomass (dry weight) (33 to 627 μg/g) (Liu et al., 2002). Gray et al. (1996) estimated a fivefold higher flux of ^{137}Cs through rhizomorphs of *Armillaria gallica* than through undifferentiated hyphae. Accumulation of ^{137}Cs occurred at the sites of initiation of basidiocarp primordia in *Schizophyllum commune* when blue light induced fructification (Gray et al., 1995). High concentrations of ^{137}Cs and lower concentrations of ^{210}Pb and ^{226}Ra have been detected in mushrooms (Kirchner and Daillant, 1998). Models have been developed to estimate the ^{210}Pb deposited onto the fructifications to the ^{210}Pb concentrations measured.

11.6.2   Biosorption by White-Rot Fungi

The success of white-rot fungi in pulp bleaching effluents has extended their role in heavy metal biosorption. White-rot fungi have the ability to sequester toxic levels of metal ions during growth in soil. The concentrations of heavy metal ions in soil and contaminated sites are generally higher than those in wood. These metals affect growth, reproduction, metabolic activity, mycelial morphology, enzymatic activities, and so on (Baldrian, 2003). On the contrary, fungi trigger defense mechanisms to deter ill effects of metals on metabolism.

In general, heavy metals cause a reduction in growth, followed by changes in morphology in all groups of fungi. Cadmium adsorption to *Schizophyllum commune* leads to the formation of aerial hyphae and loops and the development of connective filaments (Lilly et al., 1992). Certain fungi change their color in the presence of heavy metals. *Stereum hirsutum* produced a yellow-orange pigment in mycelium and extracellularly in the presence of 0.25 mM or more Cd(II) (Baldrian et al., 1996). A brown pigment was produced as a result of growth of *Trametes versicolor* on medium containing Cd(II) (Baldrian and Gabriel, 1997). *Schizophyllum commune* formed black mycelial pellets in Pb-containing media (Gabriel et al., 1994).

In general, heavy metals inhibit enzymatic reactions at several levels. Metals cause oxidative damage to proteins by binding to amino acid residues in enzymes. In white-rot fungi, the extracellular enzymes are affected at the transcriptional and translational levels. Low levels of essential metals are necessary for the production of ligninolytic enzymes. Manganese plays an important role in the expression of manganese peroxidase (MnP) and laccase and in the degradation of lignin. Copper is a cofactor for enzyme laccase. Addition of copper has a positive effect on the production of laccase in *Ceriporiopsis subvermispora* (Karahanian et al., 1998), *Trametes versicolor* (Collins and Dobson, 1997), *Pleurotus ostreatus* (Palmieri et al., 2000b), *Pleurotus sajor-caju* (Soden and Dobson, 2001), *Trametes trogii* (Levin et al., 2002), *Trametes pubescens*, *T. multicolor*, *T. hirsuta*, *T. gibbosa*, *Ganoderma applanatum*, *Polyporus ciliatus*, and *Panus tigrinus* (Galhaup and Haltrich, 2001). Copper also enhances the production of all isozymes of laccase in *P. ostreatus* (Collins and Dobson, 1997). Metal-response elements (MRE) have also been identified in laccase genes in *P. ostreatus* which interact with the Cu-responsive transcriptional factors. Copper and cadmium increase laccase activity in *P. ostreatus* (Baldrian and Gabriel, 2002). Heavy metals also have an inhibitory effect on these enzymes. In general, cellulases and hemicellulases are also inhibited by the presence of heavy metals. Metals also have an inhibitory effect on intracellular enzymes.

Metal ions accumulated in *Daedalea quercina* decrease in the preferential order Zn > Cu > Pb > Al (Gabriel et al., 1996a). *Ganoderma lucidum* follows a decrease in the toxicity of metals in the order Hg > Cd > Cu > U > Pb > Mn = Zn (Tham et al., 1999). Nonmetabolic concentration of Pb(II) by

fungal cells correlates with a two-step Cd(II) uptake (Norris and Kelly, 1997). The high sorptive capacity of resting cells implies surface biosorption and bioaccumulation and is catalyzed by enzymes responsible for binding, transporting, and depositing the metal into the vacuoles (Yetis et al., 2000). *Trametes versicolor* is a good cadmium biosorbent, as it removes all Cd(II) ions within 2 hours (Jarosz-Wilkolazka et al., 2002). This appears to be an energy-independent surface binding process that occurs at a rate of about 2 mg Cd(II) per gram of mycelial dry weight. Extracellular laccase activity was stimulated significantly when Cd(II) ions were added to 10-day-old cultures. *Pleurotus ostreatus* accumulated 20% of Cd(II) intracellularly in the presence of 150 ppm of Cd(II) in the medium (Favero et al., 1991). Some isolates of *Wolfiporia cocos* have the capacity to degrade pine wood treated with high concentrations of copper chromated arsenate (CCA) wood preservatives, and others are not able to grow even at lower concentrations (De Groot and Woodward, 1999; Woodward and De Groot, 1999). Eleven of 12 isolates of the dry-rot fungus *Serpula lacrymans* have shown tolerance to the copper-based wood preservative copper citrate (Hastrup et al., 2005). A substantial amount of work on white-rot fungi during the past decade indicates that these are good candidates for the biosorption of heavy metals (Table 11.6).

11.6.3   Biosorption by Yeasts

The order of metal affinity is U > Zn > Cd > Cu for living and nonliving brewer's yeast, Zn > (Cd) > U > Cu for nonliving baker's yeast, and Zn > Cu ~ (Cd) > U for living baker's yeast (Volesky and May-Phillips, 1995). *Saccharomyces cerevisiae* exhibits fine needlelike crystals on both the outside and inside of the cells due to U uptake. Uptake of different oxidation states of selenium [Se(IV) and Se(VI)] and antimony [Sb(III) and Sb(V)] by bakers's yeast demonstrates that not only the metal, but also its oxidation state, are important for biosorption (Perez-Corona et al., 1997).

The uptake of chromium by yeasts can be due to biosorption and active accumulation. A yeast mechanism for chromium stress may involve oxidation–reduction reactions, interaction with cellular organelle, binding by cytosolic molecules, formation of protein–DNA and Cr–DNA adducts and the formation of breaks in DNA strands and DNA–DNA cross-links. Different adsorption capacities for Cr(VI) are noted with different species of yeasts, and *Candida utilis* exhibits the highest activity (Rapoport and Muter, 1995). *C. utilis* showed a metal uptake of 7 for Cr(VI), 23 for Cu(II), 39 for Pb(II), 19 for Cd(II), and 28 mg for Zn per gram dry weight, respectively (Muter et al., 2002). The area of all sorbed individual metals did not cover all the surface of a single cell, except for Zn(II), which covers up to 168%. Living *S. cerevisiae* (Batic and Raspor, 1998) accumulated 30 mg/g dry weight of Cr(III) in the cell and *Candida intermedia* (Batic and Raspor, 2000) up to 0.45 mg/g dry weight. *S. cerevisiae* (Krauter et al., 1996) accumulated 4 mg/g

TABLE 11.6 Uptake of Heavy Metals by White-Rot Fungi

White-Rot Fungus/ Biomass Concentration	Metal/Conc.	Metal Uptake Capacity Removal (%)	pH	Eluant/ Reuse	Incubation (minutes/ hours/days)	Reference
Polyporus versicolor	Pb(II), 150 mg/l	25	6.0		6h	Yetis et al., 1998
	Ni(II), 150 mg/l	18				
	Cr(III), 150 mg/l	12				
	Cd(II), 150 mg/l	8				
	Cu(II), 150 mg/l	5				
Phanerochaete chrysosporium (4g of wet biomass)	Pb(II), 150 mg/l	25				
	Cr(III), 150 mg/l	15				
	Cu(II), 150 mg/l	10				
	Cd(II), 150 mg/l	10				
	Ni(II), 150 mg/l	9				
P. chrysosporium Resting cells Live cells Dead cells	Pb(II)	80 mg/g dry cell 9 mg/g dry cell 20 mg/g dry cell	5.0 3–4 5.0		4h	Yetis et al., 2000
P. chrysosporium (0.2g)	Pb(II), 300 mg/l, artificial wastewater Cd(II), 300 mg/l, artificial wastewater Cu(II), 300 mg/l, artificial wastewater	69.8 mg/g 23 mg/g 20.2 mg/g	6.0		6h	Say et al., 2001
P. chrysosporium	Pb(II), 50 mg/l Cd(II), 50 mg/l	12.34 mg/g 15.2 mg/g	4.5			Li et al., 2004
Fomitopsis pinicola CCBAS 535	Cd(II), 1120 mg/l	51	6.2			Gabriel et al., 1996b
P. chrysosporium	Cu(II), 100 mg/l	100	6.0	1N HCl/ 6 cycles	4h	Sing and Yu, 1998
P. versicolor	Ni(II), 500 mg/l	57 mg/g	5.0		5h	Dilek et al., 2002
P. chrysosporium	CH_3HgCl C_2H_5HgCl Hg(II), each 250 mg/l	67 mg/g 59 mg/g 51 mg/g	7.0		6h	Saglam et al., 1999

dry weight of Cr(VI), *Schizosaccharomyces pombe* (Czako-Ver et al., 1999) up to 0.95 mg/g dry weight, and *Candida utilis* (Muter et al., 2001) up to 7.2 mg/g dry weight. *Pichia guilliermondii* showed Cr(III) uptake of 10 mg/g dry weight and Cr(VI) uptake of 0.9 mg/g dry weight after 3 days (Ksheminska et al., 2003). *Candida curvata* accumulated 11.1 mg/g dry weight of Cr(III) and *Kluyveromyces lactis* 3.3 mg/g dry weight of Cr(VI). Maximum accumulation capabilities of *Pichia guilliermondii* ranged between 4.0 and 13.0 mg/g dry weight for Cr(III) and 2 to 6/7 mg/g dry weight for Cr(VI) (Kaszycki et al., 2004). The total cellular chromium is 29.3 and 52.3% for Cr(III) and Cr(VI), respectively. Uranium can also be removed by combining biosorption and precipitation using brewery yeast (Riordan et al., 1997).

The thicker mannan layer and the larger surface area of *S. cerevisiae* ATCC 834 appear to be responsible for the higher Cd(II) uptake capacity (Park et al., 2003). FTIR spectra of the protonated form and Ni(II) ion saturated yeast show that biosorption occurs primarily in the sugar and nucleic acid regions, involving —COOH and —NH groups (Padmavathy et al., 2003b). During Cd(II) biosorption by deactivated protonated yeast, the amount sorbed at equilibrium is directly proportional to the initial concentration divided by the biomass (Vasudevan et al., 2003). There is evidence of silver-containing precipitates on both the cell wall and the intracellular components within the cell (Simmons and Singleton, 1996). A method to increase silver accumulation in the industrial strain of *S. cerevisiae* has been proposed. Different yeasts isolated from the gold mining industry in Brazil bioaccumulated free and complexed silver ions (Gomes et al., 1999a). Toxic metals can be removed from an aqueous mixture containing copper, nickel, and zinc in the presence of existing co-cations (i.e., calcium and sodium ions) (Zouboulis et al., 1999). Table 11.7 shows the uptake of heavy metals by yeasts.

Crossflow filtration can be used to remove heavy metal ions from water using yeast cells of *S. cerevisiae* as carriers (Bayhan et al., 2001). The binding affinity of metal ions to yeast cells is Pb(II) > Cu(II) > Ni(II). In the presence of binary or ternary mixtures, rejection of the individual metal is reduced, except for Pb(II). Increased metal ion/yeast cell concentration reduces the total metal ion rejection in ternary mixtures.

11.6.4   Biosorption by *Aspergillus niger*

The removal of metals such as lead, copper, and cadmium from aqueous solution by *Aspergillus niger* is well recognized. Alkali-treated *A. niger* biomass binds silver to 10% of dry weight (Akthar et al., 1995). The mechanism of silver biosorption occurs through stoichiometric exchange with Ca(II) and Mg(II) of the sorbent. Analytical electron microscopy of *A. niger* biomass reveals the localization of nickel in the form of small rectangular crystals associated with the cell wall and inside the cell (Magyarosy et al., 2002). These crystals are identified as nickel oxalate dihydrate. The sorptive capacity improves in terms of the removal of lead, copper, and cadmium when the live

TABLE 11.7 Uptake of Heavy Metals by Yeasts

Yeast/Biomass Concentration	Metal/Conc.	Metal Uptake Capacity/ Removal (%)	pH	Eluant/ Reuse	Incubation (minutes/ hours/days)	Reference
Saccharomyces cerevisiae (dry biomass)	U, 0.1–0.5 M/l	84–98	4.5			Omar et al., 1996
S. cerevisiae (0.4 g dry biomass)	UO_2(II), 0.4 mM Pb(II), 0.4 mM Zn(II), 0.4 mM Cd(II), 0.4 mM Ag(II), 0.4 mM Cr(III), 0.4 mM	0.9 mM/g 0.9 mM/g 0.28 mM/g 0.36 mM/g 0.14 mM/g 0.23 mM/g	4.5	0.1 M Na_2CO_3	60 m	Omar et al., 1997
Talaromyces emersonii	U	280 mg/g	5.0		2 m	Bengtsson et al., 1995
Debaryomyces hansenii	UO_2(II), 125 mg/l	137 mg/g 343 mg/g	5.8		1 h	Tsuruta, 2002
Kluyveromyces marxianus (15 mg dry weight)						
Candida utilis	Cr(VI), 1 mg/100 ml	0.35 mg/g			15 m	Rapoport and Muter, 1995
Candida sp.	Cu(II), 100 mg/l	49 mg/l	4.0		96 h	Donmez and Aksu, 1999
S. cerevisiae		54 mg/l	4.0			
Kluyveromyces marxianus		67 mg/l	5.0			
S. cerevisiae (1 mg/cm³)	Ag(I)	225 µM/g			5 m	Singleton and Simmons, 1996
S. cerevisiae ATCC 834 (0.21 g) *S. cerevisiae* ATCC 24858 (0.38 g)	Cd(II), 100 mg/l	37.30 mg/g 18.90 mg/g	8.6		1 h	Park et al., 2003
S. cerevisiae (1 g/l)	Ni(II), 1000 mg/l	11.4 mg/g	6.75		20–60 m	Padmavathy et al., 2003a
S. cerevisiae (500 mg)	Pd(II), 25 ng/ml Pt(IV), 75 ng/ml	12 µg/g (99%) 8 µg/g (78%)	1.4–1.6 1.8–2.2		30 m 60 m	Godlewska-Zylkiewicz, 2003

A. niger biomass is pretreated with formaldehyde, dimethyl sulfoxide, sodium hydroxide, and detergent compared to live biomass (Kapoor and Viraraghavan, 1998a). Live biomass removes nickel more effectively than does alkaliboiled biomass (Kapoor et al., 1999). Lead, copper, and cadmium display a lower adsorptive capacity when present together in solution than when present as individual metal ions in solution. During growth of *A. niger* on gold mining solution containing cyano-metal complexes, gold, silver, copper, iron, and zinc are accumulated due to the presence of metal precipitation on the cell surface (Gomes et al., 1999b). *A. niger* has the capability to solubilize pyromorphite (Sayer et al., 1999). *A. niger* and *Trichoderma harzianum* also play a role in the solubilization of heavy metal sulfides (Grayston and Wainwright, 1988). Table 11.8 lists the uptake of heavy metals by *A. niger.*

11.6.4.1   Role in Soil Bioremediation.   Fungi are widespread in the natural environment and can be the dominant organisms in many soils, especially at low pH. Certain soil fungi can desorb metal ions at a wide range of pH levels. A process to bioremediate three types of soils contaminated with heavy metals has been developed using *A. niger* (Wasay et al., 1998). The fungus biomass produces weak organic acids, mostly citric acid, for the leaching of heavy metals from the contaminated soils. Several types of metals are leached at different levels in loam, clay loam, and sandy clay loam soils. These contaminated soils can be further remediated to the Province of Quebec Standards for heavy metals after 20 to 25 days. The process may be applied for in situ or ex situ mycoremediation at a cost of $5.00 per cubic meter.

11.7   FUNGAL BIOSORPTION OF HERBICIDES AND PHENOLS

Table 11.9 shows the uptake of phenols by fungi. The living mycelia of *Emericella nidulans* adsorb 15% of atrazine, 18% of 2,4-chlorophenoxyacetic acid (2,4-D), 29% of 2,4-dichlorophenol (2,4-DCP), and 30% of 4-chlorophenol (4-CP) (Benoit et al., 1998). No metabolites were detected in solutions containing atrazine, 2,4-D, and 2,4-DCP after 144 hours. Sorption of 4-CP showed the identification of 4-chlorocatechol and two unidentified compounds more polar than 4-CP, indicating biodegradation as the mechanism of action. Uptake by living mycelium in the fungal cells was due to the bioaccumulation of 4-CP, 2,4-DCP, and 2,4-D. Due to a large surface area, hyphae can aggregate to a mycelium, which can act as a biosorbent for the organic pollutants. Inactivated nonliving fungal biomass with freeze-dried preparations of both fungi shows rapid adsorption on fungal cell wall surfaces. Different species of Basidiomycetes display differences in adsorbed amounts of pentachlorophenol (Logan et al., 1994). Uptake of 5 to 18% of pentachlorophenol occurred on mycelia. A macrofungus has shown the ability to adsorb endosulfan from water (Sudhakar and Dikshit, 1999). The fungus can remove 80% of endosulfan at an initial concentration of 10mg/l after 24 hours. *Pleurotus*

TABLE 11.8 Uptake of Heavy Metals by *Aspergillus niger*

Fungus/Biomass Concentration	Metal/Conc.	Metal Uptake Capacity/ Removal (%)	pH	Eluant/Reuse	Incubation (minutes/ hours/days)	Reference
Aspergillus niger	Ni(II), 6.5 mM	98	4.75		100h	Magyarosy et al., 2002
A. niger	Cu(II), Pb(II) 50 mg/dm^3 75 mg/dm^3	51, 86 30, 69	4.5		170h	Dursun et al., 2003
A. niger (0.1 g)	Pb(II), 10 mg/l Cd(II), 10 mg/l Cu(II), 10 mg/l	7 mg/g 3 mg/g 3 mg/g	4.0	0.05 N HNO$_3$/0.1 M Ca(II), Mg(II), 0.1 N Na$_2$CO$_3$, NaHCO$_3$, NH$_4$Cl/ 5 cycles	8h	Kapoor et al., 1999

TABLE 11.9 Uptake of Phenols by Fungi

Fungus/Biomass Concentration	Phenol/Conc.	Metal Uptake Capacity/ Removal (%)	pH	Eluant/ Reuse	Incubation (hours)	Reference
Emericella nidulans Living mycelium	4-Chlorophenol, 0.11 mM 2,4-Dichlorophenol, 0.10 mM	30 29		0.01 M CaCl$_2$	144 h	Benoit et al., 1998
Nonliving biomass	2,4-Dichlorophenol	67			3 h	
Penicillium miczynski (nonliving biomass)		35				
Phanerochaete chrysosporium	Phenol, 500 mg/l *o*-Chlorophenol, 500 mg/l *p*-Clorophenol, 500 mg/l 2,4,6-Trichlorophenol, 500 mg/l	1.23 mM/g 1.49 mM/g 1.78 mM/g 2.14 mM/g	6.0	30% (v/v) Methanol/ more than 10 cycles	4 h	Denizli et al., 2004
Pleurotus sajor-caju	Phenol *o*-Chlorophenol *p*-Clorophenol 2,4,6-Trichlorophenol, each 500 mg/l	0.95 mM/g 1.24 mM/g 1.47 mM/g 1.89 mM/g	6.0	30% (v/v) Methanol/ more than 5 cycles	4 h	Denizli et al., 2005

sajor-caju removed chlorophenols within 4 hours and sorption increased with increasing pH (Denizli et al., 2005). The sorption is in the preferential order 2,4,6-trichlorophenol > *para*-chlorophenol > *ortho*-chlorophenol > phenol. *Phanerochaete chrysosporium* attains a similar affinity order with the same phenols (Denizli et al., 2004). Additional work is needed on the biosorption of herbicides and phenols, including elucidation of the mechanism of action and the process of metal biodegradation and bioaccumulation.

11.8   FUNGAL BIOSORPTION OF DYES

Some information on the biosorption of dyes was given in Chapter 10. Table 11.10 lists the color removal and biosorption capacities of various types of dyes by fungi. The dead and pulverized macrofungus *Fomitopsis carnea* displays an excellent uptake potential for all cationic dyes, and the affinity of sorbent varies with the dye (Mittal and Gupta, 1996). The bottle point method is used for adsorption equilibria tests. The first-order reaction kinetics describes the biosorption and dye transport. Sorption enhances with mixing intensity of 500 rpm and can be employed in the design of a batch reactor. A thermotolerant yeast, *Kluyveromyces marxianus* IMB3, decolorizes Remazol Black B, a vinyl sulfone type of reactive dye (Meehan et al., 2000). The maximum color removal was 98% at 37ºC and pH 5.0 within 18 hours. Addition of heavy inoculum produced rapid decolorization. The yeast biomass did not decolorize under anaerobic conditions. The kinetic and equilibrium modeling of biosorption of this dye have also been studied in a batch system by dried *Rhizopus arrhizus* (Aksu and Tezer, 2000). The fungal biomass exhibited second-order kinetics.

11.9   FUNGAL BINARY AND TERNARY BIOSORPTION SYSTEMS

Fungal biosorption of single metal is quite common, whereas the competitive uptake of the multimetal mixtures is a complex process. The mechanism of mycosorption and reversibility versus irreversibility indicates interactive effects on the surface due to the presence of many metals in solution. These interactions are antagonistic or synergistic in nature and can potentially influence the biochemical processes of fungi. Binary and ternary mycosorption involve competition of metals for the binding sites, metal combination and concentration, order of metal addition, and contact time.

11.9.1   Binary Biosorption Systems

Sorption of Ni(II) by *R. arrhizus* has been shown to be antagonistic during the combined effect of Pb(II) and Ni(II) ions (Sag and Kutsal, 1997). The

TABLE 11.10 Fungal Biosorption of Dyes

Fungus	Dye Conc.	Biosorption Capacity (mg/g)	Reference
Fomitopsis carnea	Orlamar Red BG, 50 mg/l	503	Mittal and Gupta, 1996
	Orlamar Blue G, 50 mg/l	545	
	Orlamar Red GTL, 50 mg/l	644	
Rhizopus arrhizus	Remazol Black B, 800 mg/l	588	Aksu and Tezer, 2000
Aspergillus niger	Congo Red, 50 mg/l	14.72	Fu and Viraraghavan, 2002
A. niger	Basic Blue 9, 50 mg/l	10–18	Fu and Viraraghavan, 2000
A. niger	Acid Blue 29, 50 mg/l	1–13	Fu and Viraraghavan, 1999
A. niger	Reactive Brilliant Red, 250 mg/l	14	Gallagher et al., 1997
Rhizopus oryzae		102.6	
Geotrichum fici	Reactive Black 5 dye waste	45	Polman and Breckenridge, 1996
Kluyveromyces waltii	Reactive Black 5 dye waste	72	
Pichia carsonii	Reactive Black 5 dye waste	32	
	Sulfur Black 1 dye waste	549	
Candida rugosa	Reactive Black 5 dye waste	31	
	Sulfur Black 1 dye waste	407	
Saccharomyces cerevisiae	Reactive Blue 19 dye waste	69	
Botrytis cinerea	Reactive Blue 19 dye waste	42	
	Sulfur Black 1 dye waste	360	
Tremella fuciformis	Reactive Black 5 dye waste	79	
	Sulfur Black 1 dye waste	892	
Xeromyces bisporus	Reactive Blue 19 dye waste	60	
	Sulfur Black 1 dye waste	60	

Pb(II) ions dominated competitive binding in binary metal mixtures. Sorption of Pb(II) during the interactive influences of Pb(II) and Ni(II) was synergistic at concentrations of Pb(II) ions at 10 and 25 mg/l, but at increasing concentrations of Ni(II) ions in the range 25 to 150 mg/l. Bioremoval of both Pb(II) and Ni(II) by *R. arrhizus* can be achieved in batch-stirred reactors in series at pH 4.5. The first reactor can remove a large concentration of Pb(II), and the second reactor, the residual Ni(II).

R. arrhizus has a capacity for individual biosorption of Cr(VI) and Cu(II) from binary mixtures and antagonistic uptake of these combined metals (Sag and Kutsal, 1996a). The competitive equilibrium adsorption data of Cr(VI) and Fe(III) from binary mixtures by *R. arrhizus* fit well in the competitive Langmuir model (Sag and Kutsal, 1996b). The models are applied for simultaneous adsorption of Cu(II) and Zn(II) by *R. arrhizus* (Sag et al., 1998). The binary sorption of Cr(VI) and Cu(II), and Cr(VI) and Fe(III), on free *R. arrhizus* in a packed column reactor in the continuous mode has been examined (Sag et al., 1999, 2000a). The combined action of Cr(VI) and Fe(III) displayed an antagonistic behavior, as the other metal ions reduce the maximum column capacity and adsorption yield. The column equilibrium results of Cr(VI) and Cu(II), and Cr(VI) and Fe(III), on *R. arrhizus* also correlated satisfactorily with the empirical Freundlich isotherms for the binary metal mixtures.

Experimental and model-predicted data of binary systems on *R. arrhizus* in a CFST under operating conditions have been compared (Sag et al., 2000b). The adsorption uptake of Pb(II) and Cu(II) increases with increased concentrations at a fixed concentration of the competing metal ion. Pb(II) ions dominate competitive binding during binary mixture simultaneous adsorption of Pb(II) and Cu(II). The combined action on Pb(II) and Ni(II) is antagonistic, as the removal of one metal decreases with increased concentration of the other metal. The experimental results correlate reasonably well by the model. A mathematical model based on mass balances for the liquid and solid phases has been used to predict the performance of a binary system of Cr(VI) and Fe(III) on *R. arrhizus* in a semibatch reactor (Sag et al., 2001). Forward and backward rate constants K_1 and K_2 of single metal biosorption are modeled for binary adsorption. The adsorption capacity of Cr(VI) is greater than that of Fe(III), which correlates well with the single-component data. Cr(VI) adsorbs selectively from the mixture, but the selectivity of the biomass for Fe(III) increases with an increase in the ratio of concentrations of Fe(III) and Cr(VI) ions. The biosorption capacity of an individual metal ion has been shown to decrease in the presence of other metal ions using *Mucor rouxii* biomass (Yan and Viraraghavan, 2003). Sorption of Pb(II) is lower in a two- or multimetal system. However, the total sorption capacity is increased more in three-metal ion systems than in bimetal ion systems. The sorption capacities of live *Funalia trogii* immobilized in calcium–alginate gel beads and heat-inactivated form in multimetal ions systems are in the order Hg(II) > Cd(II) > Zn(II) (Arica et al., 2004). The total sorption capacities

of the immobilized preparations in a multimetal system are lower than in a single-metal system. The Pb(II) ions are more preferential than Cd(II) ions during simultaneous sorption by a biomass of *P. chrysosporium* (Li et al., 2004).

11.9.2   Ternary Biosorption Systems

The combined effect of ternary metals on *R. arrhizus* is antagonistic (Sag and Kutsal, 1998). The equilibrium uptake of Cr(VI) by *R. arrhizus* occurs in the presence of increasing concentrations of Fe(III) and Cu(II). The Cr(VI) uptake depends on the ratio of the dominant metal ion to competing metal ions. The sorption capacity, selectivity, and biomass yield are enhanced by increasing the ratio of Cr(VI) concentration to other metal and/or to the total metal concentration. The Fe(III) ions show a more or less similar sorption pattern, although Fe(II) ions compete strongly for the binding sites. On the contrary, adsorption of Cu(II) is significantly low, due to poor affinity and low uptake at pH 2.0. A higher capability of Cr(VI) is established to other metals, which correlates reasonably well with single and binary systems.

Experimental and model-predicted data of lead, nickel, and copper concentration profiles, and the metal ratios have been compared in a ternary system on *R. arrhizus* in a CFST under optimum operating conditions (Sag et al., 2000b). Variations in the initial concentration of Pb(II) ions and the Ni(II) and Cu(II) ion concentrations constants were used. At equilibrium, adsorbed Pb(II) amount increased with an increasing Pb(II) concentration and ratio to other metal ions. Sorption of Cu(II) and Ni(II) displayed a similar pattern. Significant inhibition in metal uptake was noted in the presence of an increasing concentration of other metal ions. The equilibrium uptake was 48.79 mg Pb(II)/g, 25.78 Cu(II)/g, and 41.44 mg Ni(II)/g at constant metal concentrations. This implies a synergistic action due to metals interaction. In multimetal ternary systems using *R. arrhizus* in batch-stirred reactors, the adsorption capacities displayed the preferential order Pb(II) > Ni(II) > Cu(II) at pH 5.0 (Sag et al., 2000c). A CFST can handle large concentrations of multimetal solutions with greater biosorption yields and is applicable for the continuous treatment of large quantities of wastewaters. The multicomponent system of immobilized *Mucor rouxii* in polysulfone matrix showed capacities of 0.36, 0.31, and 0.40 mg/g for Cd, Ni, and Zn, respectively, at each initial ion concentration of 5 mg/l (Yan and Viraraghavan, 2001). Nearly 50% removal of cadmium, nickel, and zinc occurred for 8, 7, and 8 bed volumes, respectively. The majority of Cd, Ni, and Zn ions were eluted in 6, 6, and 6.5 bed volumes, respectively, using 0.05 N HNO_3, similar to a single-component system. In a ternary system, *Aspergillus niger* biomass displayed the adsorption of heavy metals in the preferential order lead > copper > cadmium (Kapoor et al., 1999). Lead adsorption was more sensitive to the presence of copper than of cadmium; cadmium adsorption, to the

presence of both copper and lead; and copper adsorption, to the presence of lead than cadmium.

Phanerochaete chrysosporium has also been employed for competitive biosorption of Cd(II), Pb(II), and Cu(II) at an initial concentration of 100 mg/l and pH 6.0 (Say et al., 2001). Equilibrium was reached after 6 hours. The adsorption capacities were 7.80 mg for Cd(II), 16.91 mg for Pb(II), and 7.57 mg for Cu(II) per gram of dry fungal biomass. The competitive adsorption capacities for these metals were lower than under noncompetitive conditions. A combination of Pb(II) or Cu(II) and chromate ions was synergistic for Cr(VI) sorption in *Candida utilis* cells (Muter et al., 2002).

11.9.3   Effect of Co-cations

In binary and ternary systems, the presence of metal ions also affects the biosorption efficiency. In ternary systems, the uptake capacity of *Saccharomyces cerevisiae* and *Penicillium chrysogenum* is halved compared to single-metal ion sorption (Bakkaloglu et al., 1998). This is due to the presence of co-cations that reduce the capacity of the biomass. Co-cations (e.g., cadmium, copper, cobalt and nickel) have certain effects on lead and zinc biosorption by *Streptoverticillium cinnamoneum* and *P. chrysogenum* in binary and multimetal systems (Puranik and Paknikar, 1999). *S. cinnamoneum* exhibits a higher adsorptive capacity than that of *P. chrysogenum* for all metals. The uptake of lead is preferred in a multimetal solution by both types of fungal biomass. All binary systems containing zinc show mutual inhibition, and an unequal inhibition is noted in systems containing lead. The respective sorption order of *S. cinnamoneum* and *P. chrysogenum* is Pb(II) > Zn(II) = Cu(II) > Cd(II) > Ni(II) > Co(II) and Pb(II) > Cu(II) > Zn(II) > Cd(II) > Ni(II) > Co(II).

11.10   BIOSORPTION OF HEAVY METAL ANIONS

Most work is performed on the removal of heavy metal cations; little is known about the uptake of anions by a fungal biomass. Chitosan beads are known to remove molybdate (MoO_4^{2-}). Chitosan is mixed with glutaraldehyde to prevent the dissolution of beads under acidic conditions. Pure and mixed cultures of fungi have been found to biodegrade metal cyanides (Barclay et al., 1998). A mixed culture of *Fusarium solani* and *Trichoderma polysporum* was obtained on tetracyanonickelate [$K_2Ni(CN)_4$] at pH 4.0. A second mixed culture of three fungi (i.e., *Fusarium oxysporum*, *Scytalidium thermophilum*, and *Penicillium miczynski*) occurred on hexacyanoferrate [$K_4Fe(CN)_6$] at pH 4.0. The culture supernatant displayed 50% removal of the total cyanide associated with the termination of growth. Cyanide was removed in 5 days by cultivation of *Fusarium* isolated on [$K_2Ni(CN)_4$] at pH 7.0 and occurred simultaneously with removal of iron on [$K_4Fe(CN)_6$] at pH 4.0 by the fungi.

Cyanide metabolism by fungi and molecular biology of cyanide degradation has been reviewed by Barclay and Knowles (2001). A pathway for the utilization of cyanide by *Fusarium solani* has also been depicted.

Initial pH, initial Fe(III)–cyanide complex anions, and biosorbent concentration play a good role in the binding capacity to *Rhizopus arrhizus* (Aksu et al., 1999). pH 13.0 was responsible for the decrease in Fe(III)–cyanide complex anions. The Langmuir, Freundlich, and Redlich–Peterson models correlated well with the equilibrium data at pH 3.0, 7.0, and 13.0, respectively. There is a good scope for the uptake of Fe(III)–cyanide complex anions in high concentrations of wastewaters, up to 2000 mg/l. A cationic surfactant-modified yeast substantially improved the removal of chromate anions (CrO_4^{2-}) from the aqueous stream (Bingol et al., 2004). Nearly 99.5% metal ions were removed from the solution at an initial concentration of 5.2 to 2.8 mg/l Cr(VI) and pH 4.5 to 5.5. More work is required on the biosorption of anions, including the mechanism.

11.11   METAL ION RESISTANCE

Some fungi have the ability to survive in an environment containing excessive concentrations of metal ions and tend to become metal-resistant. Metal ion resistance can be attributed to differences in uptake or transport of toxic metals. It is important to study the cellular and molecular mechanisms of this response and the adaptation of various fungal species to metal stress. Intracellular chelation is an established mechanism in metal tolerance. Fungal cells have certain mechanisms to maintain metal homeostasis and prevent metal toxicity. Glutathione (GSH), metal-binding peptides, metallothionein-like peptides, and sulfide ions play roles in such mechanisms. Cellular metal stress triggers the biosynthesis of some of these molecules, which are regulated via intracellular metal sensors.

Metallothioneins (MTs) are gene-encoded cysteine-rich metal-binding proteins of low molecular weight that are found in fungi, plants, microorganisms, and animals (Clemens et al., 2003). They bind with heavy metals through clusters of thiolate bonds. Several metals induce the synthesis of phytochelatins (PCs), but the formation of a phytochelatin–metal complex is studied largely with Cu(II) and Cd(II). Incorporation of copper led to the formation of Cu(I)–metallothionein in intact *Saccharomyces cerevisiae* cells (Presta and Stillman, 1997). A high concentration of Cu(II) triggered the production of an MT in *Candida glabrata* and Cd(II) stress mainly to PCs (Mehra et al., 1988, 1989). *Neurospora crassa* synthesized an MT which belongs to family 8 of the MT classes only upon copper exposure, not due to oxidative stress (Kumar et al., 2005) (www.expasy.ch/cgi-bin/lists/metallo.txt). *Heliscus lugdunensis* and *Verticillium* cf. *alboatrum* increased the GSH pool with increasing Cd(II) concentration (Jaeckel et al., 2005a). Cadmium stress responded to simultaneous induction of MT and PC followed by an increase in the GSH

level in *H. lugdunensis* (Jaeckel et al., 2005b). The MTs and PCs could be the future biomarker tools in fungi if the analytical protocols for heavy-metal chelating properties are differentiated and refined.

Acid phosphatases are described in many filamentous fungi, but little is known on their participation in the mechanisms of heavy metal resistance. *A. niger* produced extracellular and cellular acid phosphatase activities during growth in the presence or absence of copper ions (Tsekova et al., 2002). The copper uptake by mycelia was highest when the enzyme levels were maximal. About 10% of copper ions were bound intracellularly by mycelia of different age and were not removed by acid washing. Mycelium accumulates more copper and produces more acid phosphatases in the presence of higher concentration. Phosphatase overproduction and enhanced metal uptake by mycelia indicated a possible detoxification mechanism in the resistance to copper.

A high growth rate followed by a weaker death rate indicates the tolerance development in fungi. Strong growth followed by a high death rate shows poor tolerance. Heavy metal tolerance genes may be future tools for mycoremediation. *Schizosaccharomyces pombe* is a model system for the chelation and transport of cadmium (Ow, 1996). A gene *hmt1* for heavy metal resistance encoding a vacuolar membrane protein has been isolated that belongs to a member of the ATP-binding cassette (ABC) type of transporter family (Ortiz et al., 1992). Cadmium tolerance in *S. pombe* requires two purine biosynthetic enzymes (Juang et al., 1993). With the identification of the target genes in this model organism or other fungus, their sequences can be modified for heterologous or homologous expression. A total of 177 heavy metal–resistant mutants of *Trichoderma* have been isolated and tested for cross-resistance to other heavy metals (Kredics et al., 2001). Some mutants are effective on media containing the respective heavy metals.

11.12   CONCLUSIONS AND FUTURE PERSPECTIVES

The persistence of metals in the environment leads to a quest for new technologies. The unique, versatile, and innovative process of fungal biosorption started to gain importance in the 1980s. The applications highlighted above are clearly indicators of success in fungal biosorption. More information is now available on different types of fungal biomass and biosorbents for the uptake of metals. The development of metal-resistant species is still in infancy. The best combination of biomass type, environmental conditions, and choice of metals are explored sufficiently in single-metal systems. The use of dead biomass is established as a substitute for ion-exchange resins. The role of fungi in the sorption of heavy metals may lead to the development of emerging technology for the treatment of mineral-processing wastewater. A new door of opportunity for cost-effective technology will open in this century with regulatory criteria.

The suitability of metal desorption, regeneration, and multiple reuse of biomass is equally important. The mechanism of metal binding by a fungal cell wall can give new insights to optimize the process of operation. The different cell wall constituents can be manipulated chemically. Similarly, genetic manipulation of cell walls related to the uptake capacity and used to withstand high dose of metals can be exploited. This suggests a need for the development by bioengineering techniques of fungal biomass and biopolymers of increased metal capacities and specificities. Only then can the fungal biosorbents of new generation with higher specificities be produced.

Fungi can play a key role in determining the fate of metals in soil ecosystems and act as significant potential agents in the mycoremediation of metal-contaminated soils. Several species isolated from the polluted sites show excellent capabilities for metal scavenging. Fungi surviving and accumulating the metals from contaminated soils are the best targets for successful mycoremediation. Understanding translocation within the mycelium is essential for the accumulation of metals by filamentous fungi in soil. Rapid translocation makes possible an enhanced capacity for the sequestration of metals. The introduction of filamentous fungi into the contaminated soils will absorb and translocate the metals, which eventually are concentrated in the basidiocarps. Basidiocarps can then be harvested and metals are extracted for recycling or disposal. A simple and inexpensive method for in situ remediation of metal-contaminated soil by fungi will be of great benefit in the future.

The major difficulty, at present, lies in the performance of binary and ternary metal systems. Mathematical models to predict the performance of a sorptive system under different operating conditions can provide valuable knowledge to the system. At present computer models are unknown in biosorption technology, but there is a great scope for the exploitation of computer models based on metal interaction and mass transfer and hydrodynamics to optimize process design. Interdisciplinary research on proven physicochemical processes integrated with a biological process and fusion of different scientific and engineering disciplines should achieve the full potential of mycosorption.

REFERENCES

Ahmad, A., P. Mukherjee, S. Senapati, D. Mandal, et al. (2003) Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium oxysporum. Colloids Surf. B* **28**: 313–318.

Aksu, Z. (2005) Application of biosorption for the removal of organic pollutants: a review. *Process Biochem.* **40**: 997–1026.

Aksu, Z., and S. Tezer (2000) Equilibrium and kinetic modeling of biosorption of Remazol Black B by *Rhizopus arrhizus* in a batch system: effect of temperature. *Process Biochem.* **36**: 431–439.

Aksu, Z., A. Calik, A.Y. Dursun, and Z. Demircan (1999) Biosorption of iron(III)-cyanide complex anions to *Rhizopus arrhizus*: application of adsorption isotherms. *Process Biochem*. **34**: 483–491.

Akthar, N., S. Sastry, and M. Mohan (1995) Biosorption of silver ions by processed *Aspergillus niger* biomass. *Biotechnol. Lett*. **17**: 551–556.

Arica, M.Y., Y. Kacar, and O. Genc (2001) Entrapment of white-rot fungus *Trametes versicolor* in Ca–alginate beads: preparation and biosorption kinetic analysis for cadmium removal from an aqueous solution. *Bioresour. Technol*. **80**: 121–129.

Arica, M.Y., G. Bayramoglu, M. Yilmaz, S. Bektas, and O. Genc (2004) Biosorption of Hg^{2+}, Cd^{2+}, and Zn^{2+} by Ca–alginate and immobilized wood-rotting fungus *Funalia trogii*. *J. Hazard. Mater*. **109**: 191–199.

Ashkenazy, R., L. Gottlieb, and S. Yannai (1997) Characterization of acetone washed yeast biomass functional groups involved in lead biosorption. *Biotechnol. Bioeng*. **55**: 1–10.

Bag, H., A.R. Turker, M. Lale, and A. Tunceli (2000) Separation and speciation of Cr(III) and Cr(VI) with *Saccharomyces cerevisiae* immobilized on sepiolite and determination of both species in water by FAAS. *Talanta* **51**: 895–902.

Bai, R.S., and T.E. Abraham (2002) Studies on enhancement of Cr(VI) biosorption by chemically modified biomass of *Rhizopus nigricans*. *Water Res*. **36**: 1224–1236.

Bakkaloglu, I., T.J. Butter, L.M. Evison, F.S. Holland, and I.C. Hancock (1998) Screening of various types biomass for removal and recovery of heavy metals (Zn, Cu, Ni) by biosorption, sedimentation and desorption. *Water Sci. Technol*. **38**: 269–277.

Baldrian, P. (2003) Interactions of heavy metals with white-rot fungi. *Enzyme Microb. Technol*. **32**: 78–91.

Baldrian, P., and J. Gabriel (1997) Effect of heavy metals on the growth of selected wood-rotting basidiomycetes. *Folia Microbiol*. **42**: 521–523.

Baldrian, P., and J. Gabriel (2002) Copper and cadmium increase laccase activity in *Pleurotus ostreatus*. *FEMS Microbiol. Lett*. **206**: 69–74.

Baldrian, P., J. Gabriel, and F. Nerud (1996) Effect of cadmium on the ligninolytic activity of *Stereum hirsutum* and *Phanerochaete chrysosporium*. *Folia Microbiol*. **41**: 363–367.

Barclay, M., and C.J. Knowles (2001) Cyanide biodegradation by fungi. In: *Fungi in Bioremediation*, G.M. Gadd, ed. Cambridge University Press, Cambridge, pp. 335–358.

Barclay, M., A. Hart, C.J. Knowles, J.C.L. Meeussen, and V.A. Tett (1998) Biodegradation of metal cyanides by mixed and pure cultures of fungi. *Enzyme Microb. Technol*. **22**: 223–231.

Batic, M., and P. Raspor (1998) Uptake and bioaccumulation of Cr(III) in yeast *Saccharomyces cerevisiae*. *Food Technol. Biotechnol*. **36**: 291–297.

Batic, M., and P. Raspor (2000) Effect of cultivation mode on a bioprocess for chromium yeast biomass enrichment. *Pfluegers Arch. Eur. J. Physiol*. **439**(Suppl.): R73–R75.

Bayhan, Y.K., B. Keskinler, A. Cakici, M. Levent, and G. Akay (2001) Removal of divalent heavy metal mixtures from water by *Saccharomyces cerevisiae* using crossflow microfiltration. *Water Res*. **35**: 2191–2200.

Bayramoglu, G., A. Denizli, S. Bektas, and M.Y. Arica (2002) Entrapment of *Lentinus sajor-caju* into Ca-alginate gel beads for removal of Cd(II) ions from aqueous solution: preparation and biosorption kinetic analysis. *Microchem. J.* **72**: 63–76.

Bayramoglu, G., S. Bektas, and M.Y. Arica (2003) Biosorption of heavy metal ions on immobilized white-rot fungus *Trametes versicolor. J. Hazard. Mater.* **101**: 285–300.

Bengtsson, L., B. Johansson, T.J. Hackett, L. McHale, and A.P. McHale (1995) Studies on the biosorption of uranium by *Talaromyces emersonii* CBS 814.70 biomass. *Appl. Microbiol. Biotechnol.* **42**: 807–811.

Benoit, P., E. Barriuso, and R. Calvet (1998) Biosorption characterization of herbicides, 2,4-D and atrazine, and two chlorophenols on fungal mycelium. *Chemosphere* **37**: 1271–1282.

Bingol, A., H. Ucun, Y.C. Bayhan, A. Karagunduz, et al. (2004) Removal of chromate ions from aqueous stream by a cationic surfactant-modified yeast. *Bioresour. Technol.* **94**: 245–249.

Brauch, V., and E.U. Schlunder (1975) The scale-up of activation of carbon column for wastewater purification, based on results from batch tests. *Chem. Eng. Sci.* **30**: 539–548.

Butter, T.J., L.M. Evison, I.C. Hancock, and F.S. Holland (1998) The kinetics of metal uptake by microbial biomass: implications for the design of a biosorption reactor. *Water Sci. Technol.* **38**: 279–286.

Clemens, S., C. Simm, and T. Maier (2003) Heavy metal-binding proteins and peptides. In: *Polyamides and Complex Proteinaceous Materials*, S. Fahnestock and A. Steinbuchel, eds. Wiley-VCH, Weinheim, Germany, pp. 255–288.

Collins, P.J., and A.D.W. Dobson (1997) Regulation of laccase gene transcription in *Trametes versicolor. Appl. Environ. Microbiol.* **63**: 3444–3450.

Crichton, R.R. (1991) *Inorganic Biochemistry of Iron Metabolism*. Ellis Horwood, Chichester, UK.

Czako-Ver, K., M. Batic, P. Raspor, M. Sipiczki, and M. Pesti (1999) Hexavalent chromium uptake by sensitive and tolerant mutants of *Schizosaccharomyces pombe. FEMS Microbiol. Lett.* **178**: 109–115.

De Groot, R.C., and B. Woodward (1999) Using copper-tolerant fungi to biodegrade wood treated with copper-based preservatives. *Int. Biodeterior. Biodegrad.* **44**: 17–27.

Denizli, A., N. Cihangir, A.Y. Rad, M. Taner, and G. Alsancak (2004) Removal of chlorophenols from synthetic solutions using *Phanerochaete chrysosporium. Process Biochem.* **39**: 2025–2030.

Denizli A., N. Cihangir, N. Tuzmen, and G. Alsancak (2005) Removal of chlorophenols from aquatic systems using the dried and dead fungus *Pleurotus sajor caju. Bioresour. Technol.* **96**: 59–62.

Dias, M.A., I.C.A. Lacerda, P.F. Pimentel, H.F. de Castro, and C.A. Rosa (2002) Removal of heavy metals by an *Aspergillus terreus* strain immobilized in a polyurethane matrix. *Lett. Appl. Microbiol.* **34**: 46–50.

Dilek, F.B., A. Erbay, and U. Yetis (2002) Ni(II) biosorption by *Polyporus versicolor. Process Biochem.* **37**: 723–726.

Donmez, G., and Z. Aksu (1999) The effect of copper(II) on the growth and bioaccumulation properties of some yeasts. *Process Biochem.* **35**: 135–142.

Dursun, A.Y., G. Ulsu, Y. Cuci, and Z. Aksu (2003) Bioaccumulation of copper(II), lead(II) and chromium(VI) by growing *Aspergillus niger. Process Biochem.* **38**: 1647–1651.

Eccles, H. (1995) Removal of heavy metals from effluent streams: why select a biological process? *Int. Biodeterior. Biodegrad.* **35**: 5–16.

Errasquin, E.L., and C. Vazquez (2003) Tolerance of uptake of heavy metals by *Trichoderma atroviride* isolated from sludge. *Chemosphere* **50**: 137–143.

Faust, S.D., and O.M. Aly (1987) Batch systems fixed fluidized. In: *Adsorption Processes for Water Treatment.* Butterworth, Boston, pp. 143–165.

Favero, N., P. Costa, and M.L. Massimino (1991) In vitro uptake of cadmium by basidiomycete *Pleurotus ostreatus. Biotechnol. Lett.* **13**: 701–704.

Fogarty, R.V., and J.M. Tobin (1996) Fungal melanins and their interactions with metals. *Enzyme Microb. Technol.* **19**: 311–317.

Franz, A., W. Burgstaller, and F. Schinner (1991) Leaching with *Penicillium simplicissimum*: influence of metals and buffers on proton extrusion and citric acid production. *Appl. Environ. Microbiol.* **57**: 769–774.

Fu, Y., and T. Viraraghavan (1999) Publications removal of Acid Blue 29 from an aqueous solution by fungus *Aspergillus niger.* In: *Proceedings of the 31st Mid-Atlantic Industrial and Hazardous Waste Conference*, Storrs, CT, N. Nikolaidis, C. Erkey, and B.F. Smets, eds. Technomic Publishing Company, Lancaster, PA, pp. 510–519.

Fu, Y., and T. Viraraghavan (2000) Removal of a dye from an aqueous solution by fungus *Aspergillus niger. Water Qual. Res. J. Can.* **35**: 95–111.

Fu, Y., and T. Viraraghavan (2002) Removal of Congo Red from an aqueous solution by fungus *Aspergillus niger. Adv. Environ. Res.* **7**: 239–247.

Gabriel, J., M. Mokrejs, J. Bily, and P. Rychlovsky (1994) Accumulation of heavy metals by some wood-rotting fungi. *Folia Microbiol.* **39**: 115–118.

Gabriel, J., O. Kofronova, P. Rychlovsky, and M. Krenzelok (1996a) Accumulation and effect of cadmium in the wood-rotting basidiomycete *Daedalea quercina. Bull. Environ. Contam. Toxicol.* **57**: 383–390.

Gabriel, J., J. Vosahlo, and P. Baldrin (1996b) Biosorption of cadmium to mycelial pellets of wood-rotting fungi. *Biotechnol. Lett.* **10**: 345–348.

Gadd, G.M. (1993) Microbial formation and transformation of organometallic and organometalloid compounds. *FEMS Microbiol. Rev.* **11**: 297–316.

Gadd, G.M. (2001) Metal transformations. In: *Fungi in Bioremediation*, G.M. Gadd, ed. Cambridge University Press, Cambridge, pp. 359–382.

Galhaup, C., and D. Haltrich (2001) Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper. *Appl. Microbiol. Biotechnol.* **56**: 225–232.

Gallagher, K.A., M.G. Healy, and S.J. Allen (1997) Biosorption of synthetic dye and metal ions from aqueous effluents using fungal biomass. In: *Global Environmental Biotechnology*, D.L. Wise, ed. Elsevier, Amsterdam, The Netherlands, pp. 27–50.

Galun, M., P. Keller, D. Malki, H. Fedstein, E. Galun, S. Siegel, and B. Siegel (1983) Recovery of uranium(VI) from solution using precultured *Penicillium* biomass. *Water Air Soil Pollut.* **20**: 221–232.

Gardea-Torresdey, J., I. Cano-Aguilera, R. Webb, K.J. Tiemann, and F. Gutierrez-Corona (1996) Copper adsorption by inactivated cells of *Mucor rouxii*: effect of esterification of carboxyl groups. *J. Hazard. Mater.* **48**: 171–180.

Genc, O., Y. Yalcinkaya, E. Buyuktuncel, A. Denizli, M.Y. Arica, and S. Bektas (2003) Uranium recovery by immobilized and dried powdered biomass: characterization and comparison. *Int. J. Min. Process.* **68**: 93–107.

Gharieb, M.M., S.C. Wilkinson, and G.M. Gadd (1995) Reduction of selenium oxyanions by unicellular, polymorphic and filamentous fungi: cellular location of reduced selenium and implications for tolerance. *J. Ind. Microbiol.* **14**: 300–311.

Gharieb, M.M., M. Kierans, and G.M. Gadd (1999) Transformation and tolerance of tellurite by filamentous fungi: accumulation, reduction, and volatilization. *Mycol. Res.* **103**: 299–305.

Godlewska-Zylkiewicz, B. (2003) Biosorption of platinum and palladium for their separation/preconcentration prior to graphite furnace atomic absorption spectrometric determination. *Spectrochim. Acta B* **58**: 1531–1540.

Gomes, N.C.M., C.A. Rosa, P.F. Pimentel, V.R. Linardi, and L.C.S. Mendonca-Hagler (1999a) Uptake of free and complexed silver ions by yeasts isolated from a gold mining industry in Brazil. *J. Gen. Appl. Microbiol.* **45**: 121–124.

Gomes, N.C.M., M.M. Figueira, E.R.S. Camargos, L.C.S. Mendonca-Hagler, J.C.T. Dias, and V.R. Linardi (1999b) Cyano-metal complexes uptake by *Aspergillus niger. Biotechnol. Lett.* **21**: 487–490.

Gorobets, S., O. Gorobets, A. Ukrainetz, T. Kasatkina, and I. Goyko (2004) Intensification of the process of copper ions by yeast of *Saccharomyces cerevisiae* 1968 by means of a permanent magnetic field. *J. Magn. Magn. Mater.* **272**–**276**: 2413–2414.

Gray, S.N., J. Dighton, S. Olsson, and D.H. Jennings (1995) Real-time measurement of uptake and translocation of ^{137}Cs within mycelium of *Schizophyllum commune* Gr. by autoradiography followed by quantitative image analysis. *New Phytol.* **129**: 449–465.

Gray, S.N., J. Dighton, and D.H. Jennings (1996) The physiology of basidiomycete linear organs. III. Uptake and translocation of radiocaesium within differentiated mycelia of *Armillaria* spp. growing in microcosms and in the field. *New Phytol.* **132**: 471–482.

Grayston, S.J., and M. Wainwright (1988) Role of fungi in the solubilization of heavy metal sulphides. In: *Proceedings of the International Symposium on Biohydrometallurgy*, July 12–16, 1987, Warwick, Coventry, UK, P.R. Norris and D.P. Kelly, eds., pp. 552–553.

Green, F., III, and C.A. Clausen (2003) Copper tolerance of brown-rot fungi: time course of oxalic acid production. *Int. Biodeterior. Biodegrad.* **51**: 145–149.

Hafez, N., A.S. Abdel-Razek, and M.B. Hefez (1997) Accumulation of some heavy metals on *Aspergillus flavus. J. Chem. Tech. Biotechnol.* **68**: 19–22.

Hastrup, A.C.S., F. Green III, C.A. Clausen, and B. Jensen (2005) Tolerance of *Serpula lacrymans* to copper-based wood preservatives. *Int. Biodeterior. Biodegrad.* **56**: 173–177.

Helfferich, F.G. (1967) Multicomponent ion exchange in fixed beds: generalized equilibrium theory for systems with constant separation factors. *Ind. Eng. Chem. Fundam.* **6**: 362–364.

Huang, C., and C.P. Huang (1996) Application of *Aspergillus oryzae* and *Rhizopus oryza*e for Cu(II) removal. *Water Res.* **9**: 1985–1990.

Huang, C., C.P. Huang, A.L. Morehart, and D.C. Westman (1989) *Removal of Toxic Heavy Metals from Contaminated Groundwater by a Fungal Adsorption Process.* Final Technical Report. Contract 14-08-0001-G1292, U.S. Geological Survey, Washington, DC, 121 pp.

Humar, M., M. Bokan, S.A. Amartey, M. Sentjurc, P. Kalan, and F. Pohleven (2004) Fungal bioremediation of copper, chromium and boron treated wood as studied by electron paramagmetic resonance. *Int. Biodeterior. Biodegrad.* **53**: 25–42.

Huysmans, K.D., and W.T. Frankenberger (1991) Evolution of trimethylarsine by a *Penicillium* sp. isolated from agricultural evaporation pond water. *Sci. Total Environ.* **105**: 13–28.

Jaeckel, P., G.-J. Krauss, and G. Krauss (2005a) Cadmium and zinc response of the fungi *Heliscus lugdunensis* and *Verticillium* cf. *alboatrum* isolated from highly polluted water. *Sci. Total Environ.* **346**: 274–279.

Jaeckel, P., G. Krauss, S. Menge, et al. (2005b) Cadmium induces a novel metallo-thionein and phytochelatin 2 in an aquatic fungus. *Biochem. Biophys. Res. Commun.* **333**: 150–155.

Jarosz-Wilkolazka, A., and G.M. Gadd (2003) Oxalate production by wood-rotting fungi growing in toxic metal-amended medium. *Chemosphere* **52**: 541–547.

Jarosz-Wilkolazka, A., E. Malarczyk, J. Pirszel, T. Skowronski, and A. Leonowicz (2002) Uptake of cadmium ions in white-rot fungus *Trametes versicolor*: effect of Cd(II) ions on the activity of laccase. *Cell Biol. Int.* **26**: 605–613.

Juang, R.H., K.F. McCue, and D.W. Ow (1993) Two purine biosynthetic enzymes that are required for cadmium tolerance in *Schizosaccharomyces pombe* utilize cysteine sulfinate in vitro. *Arch. Biochem. Biophys.* **304**: 392–401.

Kacar, Y., C. Arpa, S. Tan, A. Denizli, O. Genc, and M.Y. Arica (2002) Biosorption of Hg(II) and Cd(II) from aqueous solutions: comparison of biosorptive capacity of alginate and immobilized live and heat inactivated *Phanerochaete chrysosporium*. *Process Biochem.* **37**: 601–610.

Kapoor, A., and T. Viraraghavan (1998a) Biosorption of heavy metals on *Aspergillus niger*: effect of pretreatment. *Bioresour. Technol.* **63**: 109–113.

Kapoor, A., and T. Viraraghavan (1998b) Removal of heavy metals from aqueous solutions using immobilized fungal biomass in continuous mode. *Water Res.* **32**: 1968–1977.

Kapoor, A., T. Viraraghavan, and D.R. Cullimore (1999) Removal of heavy metals using the fungus *Aspergillus niger*. *Bioresour. Technol.* **70**: 95–104.

Karahanian, E., G. Corsini, S. Lobos, and R. Vicuna (1998) Structure and expression of a laccase gene from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. *Biochim. Biophys. Acta* **1443**: 65–74.

Karna, R.R., L.S. Sajani, and P.M. Mohan (1996) Bioaccumulation and biosorption of Co^{2+} by *Neurospora crassa*. *Biotechnol. Lett.* **18**: 1205–1208.

Kaszycki, P., D. Fedorovych, H. Ksheminska, et al. (2004) Chromium accumulation by living yeast at various environmental conditions. *Microbiol. Res.* **159**: 11–17.

Khoo, K.-M., and Y.-P. Ting (2001) Biosorption of gold by immobilized fungal biomass. *Biochem. Eng. J.* **8**: 51–59.

Kierans, M., A.M. Staines, H. Bennett, and G.W. Gadd (1991) Silver tolerance and accumulation in yeasts. *BioMetals* **4**: 100–106.

Kirchner, G., and O. Daillant (1998) Accumulation of 210Pb, 226Ra and radioactive cesium by fungi. *Sci. Total Environ.* **222**: 63–70.

Klein, G., D. Tondeur, and T. Vermeulen (1967) Multicomponent ion exchange in fixed beds. General properties of equilibrium systems. *Ind. Eng. Chem. Fundam.* **6**: 339–350.

Krauter, P., R. Martinelli, K. Williams, and S. Martins (1996) Removal of Cr(VI) from ground water by *Saccharomyces cerevisiae*. *Biodegradation* **7**: 277–286.

Kredics, L., Z. Antal, L. Manczinger, and E. Nagy (2001) Breeding of mycoparasite *Trichoderma* strains for heavy metal resistance. *Lett. Appl. Microbiol.* **33**: 112–116.

Krishna, K.R., and L. Philip (2005) Bioremediation of Cr(VI) in contaminated soils. *J. Hazard. Mater.* **121**: 109–117.

Krogh, N., J. Olsen, B. Jensen, and M. Reeslev (1998) Uptake of Zn^{2+} by yeast and mycelial growth form of *Aureobasidium pullulans* grown in chemostat culture. *FEMS Microbiol. Lett.* **163**: 249–253.

Ksheminska, H., A. Jaglarz, D. Fedorovych, et al. (2003) Bioremediation of chromium by the yeast *Pichia guilliermondii*: toxicity and accumulation of Cr(III) and Cr(VI) and the influence of riboflavin on Cr tolerance. *Microbiol. Res.* **158**: 59–67.

Kumar, K.S., D. Dayananda, and C. Subramanyam (2005) Copper alone, but not oxidative stress, induces copper-metallothionein gene in *Neurospora crassa*. *FEMS Microbiol. Lett.* **242**: 45–50.

Lebeau, T., D. Bagot, K. Jezequel, and B. Fabre (2002) Cadmium adsorption by free and immobilized microorganisms cultivated in a liquid soil extract medium: effects of Cd, pH and techniques of culture. *Sci. Total Environ.* **291**: 73–83.

Lesuisse, E., and P. Labbe (1989) Reductive and non-reductive mechanisms of iron assimilation by the yeast *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **135**: 257–263.

Levin, L., F. Forchiassin, and L. Papinutti (2002) Effect of copper on the ligninolytic activity of *Trametes trogii*. *Int. Biodeterior. Biodegrad.* **49**: 60.

Li, Q., S. Wu, G. Liu, X. Liao, et al. (2004) Simultaneous biosorption of cadmium(II) and lead(II) ions by pretreated biomass of *Phanerochaete chrysosporium*. *Sep. Purif. Technol.* **34**: 135–142.

Liao, J., Y. Yang, S. Luo, N. Liu, J. Jin, T. Zhang, and P. Zhao (2004) Biosorption of americium-241 by immobilized *Rhizopus arrhizus*. *Appl. Radiat. Isot.* **60**: 1–5.

Lilly, W.W., G.J. Wallweber, and T.A. Lukefahr (1992) Cadmium absorption and its effects on growth and mycelial morphology of the basidiomycete fungus, *Schizophyllum commune*. *Microbios* **72**: 227–237.

Liu, N., Y.Y. Yang, S.Z. Luo, T.M. Zhang, J.N. Jin, J.L. Liao, and X.F. Hua (2002) Biosorption of Am-241 by *Rhizopus arrhizus*: preliminary investigation and evaluation. *Appl. Radiat. Isot.* **57**: 139–143.

Lo, W., H. Chua, K.-H. Lam, and S.-P. Bi (1999) A comparative investigation on the biosorption of lead by filamentous fungal biomass. *Chemosphere* **39**: 2723–2736.

Logan, B.E., B.C. Alleman, G.L. Amy, and R.L. Gilbertson (1994) Adsorption and removal of pentachlorophenol by white rot fungi in batch cultures. *Water Res.* **28**: 1533–1538.

Magyarosy A., R.D. Laidlaw, R. Kilaas, C. Echer, D.S. Clark, and J.D. Keasling (2002) Nickel accumulation and nickel oxalate precipitation by *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* **59**: 382–388.

Malik, A. (2004) Metal bioremediation through growing cells. *Environ. Int.* **30**: 261–278.

Mapolelo, M., and N. Torto (2004) Trace enrichment of metal ions in aquatic environments by *Saccharomyces cerevisiae*. *Talanta* **64**: 39–47.

Massaccesi, G., M.C. Romero, M.C. Cazau, and A.M. Bucsinszky (2002) Cadmium removal capacities of flamentous soil fungi isolated from industrially polluted sediments, in La Plata (Argentina). *World J. Microbiol. Biotechnol.* **18**: 817–820.

Matheickal, J.T., and Q. Yu (1997) Biosorption of lead(II) from aqueous solution by *Phellinus badius*. *Min. Eng.* **10**: 947–957.

Mckay, G., and M.J. Bino (1990) Fixed bed adsorption for the removal of pollutants from water. *Environ. Pollut.* **6**: 33–53.

Meehan, C., I.M. Banat, G. McMullan, P. Nigam, F. Smyth, and R. Marchant (2000) Decolorization of Remazol Black-B using a thermotolerant yeast, *Kluyveromyces marxianus* IMB3. *Environ. Int.* **26**: 75–79.

Mehra, R.K., E.B. Tarbet, W.R. Gray, and D.R. Winge (1988) Metal-specific synthesis of two metallothioneins and γ-glutamyl peptides in *Candida glabrata*. *Proc. Natl. Acad. Sci. USA* **85**: 8815–8819.

Mehra, R.K., J.R. Garey, T.R. Butt, W.R. Gray, and D.R. Winge (1989) *Candida glabrata* metallothioneins: cloning and sequence of the genes and characterization of proteins. *J. Biol. Chem.* **264**: 19747–19753.

Michelot, D., E. Siobud, J.-C. Dore, C. Viel, and F. Poirier (1998) Update on metal content profiles in mushrooms: toxicological implications and tentative approach to the mechanisms of bioaccumulation. *Toxicon* **36**: 1997–2012.

Mittal, A.K., and S.K. Gupta (1996) Biosorption of cationic dyes by dead macro fungus *Fomitopsis carnea*: batch studies. *Water Sci. Technol.* **34**: 81–87.

Mogollon, L., R. Rodriguez, W. Larrota, N. Ramirez, and R. Torres (1998) Biosorption of nickel using filamentous fungi. *Appl. Biochem. Biotechnol.* **70–72**: 593–601.

Murugesan, G.S., M. Sathishkumar, and K. Swaminathan (2006) Arsenic removal from groundwater by pretreated waste tea fungal biomass. *Bioresour. Technol.* **97**: 483–487.

Muter, O., A. Patmalniek, and A. Rapoport (2001) Interactions of the yeast *Candida utilis* and Cr(VI): metal reduction and its distribution in the cell and medium. *Process Biochem.* **36**: 963–970.

Muter, O., I. Lubinya, D. Millers, L. Grigorjeva, E. Ventinya, and A. Rapoport (2002) Cr(VI) sorption by intact and dehydrated *Candida utilis* cells in the presence of other metals. *Process Biochem.* **38**: 123–131.

Niu, H., X.S. Xu, J.H. Wang, and B. Volesky (1993) Removal of lead from aqueous solution by *Penicillium* biomass. *Biotechnol. Bioeng.* **42**: 785–787.

Norris, P.R., and D.P. Kelly (1997) Accumulation of cadmium and cobalt by *Saccharomyces cerevisiae. J. Gen. Microbiol.* **99**: 785–787.

Omar, N.B., M.L. Merroun, A.T. Gonzalez-Munoz, and J.M. Arias (1996) Brewery yeast as a biosorbent for uranium. *J. Appl. Bacteriol.* **81**: 283–287.

Omar, N.B., M.L. Merroun, J.M. Arias Penalver, and M.T. Gonzalez-Munoz (1997) Comparative heavy metal biosorption study of brewery yeast and *Myxococcus xanthus* biomass. *Chemosphere* **35**: 2277–2283.

Ortiz, D.F., L. Kreppel, D.M. Speiser, G. Scheel, G. McDonald, and D.W. Ow (1992) Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. *EMBO J.* **11**: 3491–3499.

Ow, D.W. (1996) Heavy metal tolerance genes: prospective tools for bioremediation. *Resour. Conserv. Recycl.* **18**: 135–149.

Padmavathy, V., P. Vasudevan, and S.C. Dhingra (2003a) Biosorption of nickel(II) ions on baker's yeast. *Process Biochem.* **38**: 1389–1395.

Padmavathy, V., P. Vasudevan, and S.C. Dhingra (2003b) Thermal and spectroscopic studies on sorption of nickel(II) ions on protonated baker's yeast. *Chemosphere* **52**: 1807–1817.

Paknikar, K.M., P.R. Puranik, A.D. Agate, and S.R. Naik (1998) Metal biosorbents from waste fungal biomass: a new bioremedial material for control of heavy metal pollution. In: *Bioremediation: Principles and Practices, Bioremediation Technologies*, S.K. Sikdar and R.T. Irvine, eds. Technomic Publishing Company, Lancaster, PA, Vol. III, pp. 557–576.

Palmieri, M.C., O. Garcia, Jr., and P. Melnikov (2000a) Neodymium biosorption from acidic solutions in batch system. *Process Biochem.* **36**: 441–444.

Palmieri, G., P. Giardina, C. Bianco, B. Fontanella, and G. Sannia (2000b) Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus. Appl. Environ. Microbiol.* **66**: 920–924.

Park, J.K., J.W. Lee, and J.Y. Jung (2003) Cadmium uptake capacity of two strains of *Saccharomyces cerevisiae* cells. *Enzyme Microb. Technol.* **33**: 371–378.

Park, D., Y.-S. Yun, J. H. Jo, and J.M. Park (2005) Mechanism of hexavalent chromium removal by dead fungal biomass of *Aspergillus niger. Water Res.* **39**: 533–540.

Perez-Corona, T., Y. Madrid, and C. Camara (1997) Evaluation of selective uptake of selenium [Se(IV) and Se(VI)] and antimony [Sb(III) and Sb(V)] species by baker's yeast cells (*Saccharomyces cerevisiae*). *Anal. Chim. Acta* **345**: 249–255.

Pethkar, A.V., S.K. Kulkarni, and K.M. Paknikar (2001) Comparative studies on metal biosorption by two strains of *Cladosporium cladosporioides. Bioresour. Technol.* **80**: 211–215.

Pighi, L., T. Pumpel, and F. Schinner (1989) Selective accumulation of silver by fungi. *Biotechnol. Lett.* **11**: 275–280.

Pillichshammer, M., T. Pumpel, R. Poder, K. Eller, J. Klima, and F. Schinner (1995) Biosorption of chromium to fungi. *BioMetals* **8**: 117–121.

Polman, J.K., and C.R. Breckenridge (1996) Biomass-mediated binding and recovery of textile dyes from waste effluents. *Text. Chem. Colorist* **28**: 31–35.

Prakasham, R.S., J.S. Merrie, R. Sheela, N. Saswathi, and S.V. Ramakrishna (1999) Biosorption of chromium VI by free and immobilized *Rhizopus arrhizus. Environ. Pollut.* **104**: 421–427.

Presta, A., and M.J. Stillman (1997) Incorporation of copper into the yeast *Saccharomyces cerevisiae*: identification of Cu(I)-Metallothionein in intact yeast cells. *J. Inorg. Biochem.* **66**: 231–240.

Price, M.S., J.J. Classen, and G.A. Payne (2001) *Aspergillus niger* absorbs copper and zinc from swine wastewater. *Bioresour. Technol.* **77**: 41–49.

Puranik, P.R., and K.M. Paknikar (1999) Influence of co-cations on biosorption of lead and zinc: a comparative evaluation in binary and multimetal systems. *Bioresour. Technol.* **70**: 269–276.

Rapoport, A.I., and O.A. Muter (1995) Biosorption of hexavalent chromium by yeasts. *Process Biochem.* **30**: 145–149.

Rashmi, K., T.N. Sowjanya, P.M. Mohan, V. Balaji, and G. Venkateswaran (2004) Bioremediation of ^{60}Co from simulated spent decontamination solutions. *Sci. Total Environ.* **328**: 1–14.

Riordan, C., M. Bustard, R. Putt, and A.P. McHale (1997) Removal of uranium from solution using residual brewery yeast: combined biosorption and precipitation. *Biotechnol. Lett.* **19**: 385–387.

Ross, I.S. (1994) Uptake of zinc by fungi. In: *Metal Ions in Fungi*, G. Winkelmann and D.R. Winge, eds. Marcel Dekker, New York, pp. 237–257.

Sabie, F.T., and G.M. Gadd (1990) Effect of zinc on the yeast–mycelium transition of *Candida albicans* and examination of zinc uptake at different stages of growth. *Mycol. Res.* **94**: 952–958.

Sag, Y., and T. Kutsal (1995) Copper(II) and nickel(II) adsorption by *Rhizopus arrhizus* in batch stirred reactors in series. *Chem. Eng. J.* **58**: 265–273.

Sag, Y., and T. Kutsal (1996a) The selective biosorption of chromium(VI) and copper(II) from binary mixtures by *Rhizopus arrhizus. Process Biochem.* **31**: 561–572.

Sag, Y., and T. Kutsal (1996b) Fully competitive biosorption of chromium(VI) and iron(III) from binary mixtures by *Rhizopus arrhizus*: use of the competitive Langmuir model. *Process Biochem.* **31**: 573–585.

Sag, Y., and T. Kutsal (1997) The simultanous biosorption process of lead(II) and nickel(II) on *Rhizopus arrhizus. Process Biochem.* **32**: 591–597.

Sag, Y., and T. Kutsal (1998) The simultanous biosorption of Cr(VI) and Fe(III) and Cu(II) on *Rhizopus arrhizus. Process Biochem.* **33**: 571–579.

Sag, Y., and T. Kutsal (2000) Determination of the biosorption activation energies of heavy metal ions on *Zoogloea ramigera* and *Rhizopus arrhizus. Process Biochem.* **35**: 801–807.

Sag, Y., A. Kaya, and T. Kutsal (1998) The simultaneous biosorption of Cu(II) and Zn on *Rhizopus arrhizus*: application of the adsorption models. *Hydrometallurgy* **50**: 297–314.

Sag, Y., I. Atacoglu, and T. Kutsal (1999) Simultaneous biosorption of chromium(VI) and copper(II) on *Rhizopus arrhizus* in packed column reactor: application of the competitive Freundlich model. *Sep. Sci. Technol.* **34**: 3155–3171.

Sag, Y., I. Atacoglu, and T. Kutsal (2000a) Equilibrium parameters for the single- and multicomponent biosorption of Cr(VI) and Fe(III) on *R. arrhizus* in a packed column. *Hydrometallurgy* **55**: 165–179.

Sag, Y., A. Yalcuk, and T. Kutsal (2000b) Mono and multi-component biosorption of heavy metal ions on *Rhizopus arrhizus* in a CFST. *Process Biochem.* **35**: 787–799.

Sag, Y., A. Kaya, and T. Kutsal (2000c) Biosorption of lead(II), nickel(II), and copper(II) on *Rhizopus arrhizus* from binary and ternary mixtures. *Sep. Sci. Technol.* **35**: 2601–2617.

Sag, Y., A. Yalcuk, and T. Kutsal (2001) Use of a mathematical model for prediction of the performance of the simultaneous biosorption of Cr(VI) and Fe(III) on *Rhizopus arrhizus* in a semi-batch reactor. *Hydrometallurgy* **59**: 77–87.

Saglam, N., R. Say, A. Denizli, S. Patir, and M.Y. Arica (1999) Biosorption of inorganic mercury and alkylmercury species on to *Phanerochaete chrysosporium* mycelium. *Process Biochem.* **34**: 725–730.

Saiano, F., M. Ciofalo, S.O. Cacciola, and S. Ramirez (2005) Metal ion adsorption by *Phomopsis* sp. biomaterial in laboratory experiments and real wastewater treatment. *Water Res.* **39**: 2273–2280.

Say, R., A. Denizli, and M.Y. Arica (2001) Biosorption of cadmium(II), lead(II) and copper(II) with the filamentous fungus *Phanerochaete chrysosporium*. *Bioresour. Technol.* **76**: 67–70.

Sayer, J.A., S.L. Raggett, and G.M. Gadd (1995) Solubilization of insoluble metal compounds by soil fungi: development of a screening method for solubilizing ability and metal tolerance. *Mycol. Res.* **99**: 987–993.

Sayer, J.A., J.D. Cotter-Howells, C. Watson, S. Hillier, and G.M. Gadd (1999) Lead mineral transformation by fungi. *Curr. Biol.* **9**: 691–694.

Schmitt, H.W., and H. Sticher (1991) Heavy metal compounds in soil. In: *Metals and Their Compounds in the Environment: Occurrence, Analysis and Biological Relevance*, E. Merian, ed. VCH, Weinheim, Germany, pp. 311–331.

Schreferl, G., C.P. Kubicek, and M. Rohr (1986) Inhibition of citric acid accumulation by manganese ions in *Aspergillus niger* mutants with reduced citrate control of phosphofructokinase. *J. Bacteriol.* **165**: 1019–1022.

Siegel, S.M., M. Galun, and B.Z. Siegel (1990) Filamentous fungi as metal biosorbents: a review. *Water Air Soil Pollut.* **53**: 335–344.

Simmons, P., and I. Singleton (1996) A method to increase silver biosorption by an industrial strain of *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **45**: 278–285.

Sing, C., and J. Yu (1998) Copper adsorption and removal from water by living mycelium of white-rot fungus *Phanerochaete chrysosporium*. *Water Res.* **32**: 2746–2752.

Singleton, I., and P. Simmons (1996) Factors affecting silver biosorption by an industrial strain of *Saccharmyces cerevisiae*. *J. Chem. Technol. Biotechnol.* **65**: 21–28.

Singleton, I., and J.M. Tobin (1996) Fungal interactions with metals and radionuclides for environmental bioremediation. In: *British Mycological Society Symposium Volume: Fungi and Environmental Change*, G.M. Gadd, N. Magan, and J.M. Frankland, eds. Cambridge University Press, Cambridge, pp. 282–298.

Smith, D.G. (1974) Tellurite reduction in *Schizosaccharomyces pombe*. *J. Gen. Microbiol.* **83**: 389–392.

Soden, D.M., and A.D.W. Dobson (2001) Differential regulation of laccase gene expression in *Pleurotus sajor-caju. Microbiology* **147**: 1755–1763.

Stoll, A., and J.R. Duncan (1997) Comparison of the heavy metal sorptive properties of three types of immobilized, non-viable *Saccharomyces cerevisiae* biomass. *Process Biochem.* **32**: 467–472.

Sudhakar, Y., and A.K. Dikshit (1999) Adsorbent selection for endosulfan removal from water environment. *J. Environ. Sci. Health B* **34**: 97–118.

Suh, J.H., D.S. Kim, J.W. Yun, and S.K. Song (1998) Process of Pb^{2+} accumulation in *Saccharomyces cerevisiae. Biotechnol. Lett.* **20**: 153–156.

Sukla, L.B., R.N. Kar, and V. Panchanadikar (1992) Leaching of copper converter slag with *Aspergillus niger* culture filtrate. *BioMetals* **5**: 169–172.

Taboski, M.A.S., T.G. Rand, and A. Piorko (2005) Lead and cadmium uptake in the marine fungi *Corollospora lacera* and *Monodictys pelagica. FEMS Microbiol. Ecol.* **53**: 445–453.

Tamaki, S., and W.T. Frankenberger (1992) Environmental biochemistry of arsenic. *Rev. Environ. Contam. Toxicol.* **124**: 79–110.

Tan, H.K.S., and I.H. Spinner (1994) Multicomponent ion exchange column dynamics. *Can. J. Chem. Eng.* **72**: 330–341.

Tezuka, T., and Y. Takasaki (1988) Biodegradation of phenylmercuric acetate by organomercury-resistant *Penicillium* sp. MR-2. *Agric. Biol. Chem.* **52**: 3183–3185.

Tham, L.X., S. Matsuhashi, and T. Kume (1999) Responses of *Ganoderma lucidum* to heavy metals. *Mycoscience* **40**: 209–213.

Thomas, H.G. (1948) Chromatography: a problem in kinetics. *Ann. N.Y. Acad. Sci.* **49**: 161–182.

Thompson-Eagle, E.T., and W.T. Frankenberger (1992) Bioremediation of soils contaminated with selenium. In: *Advances in Soil Science*, R. Lal and B.A. Stewart, eds. Springer-Verlag, New York, Vol. 17, pp. 261–309.

Thompson-Eagle, E.T., W.T. Frankenberger, and K.E. Longley (1991) Removal of selenium from agricultural drainage water through soil microbial transformations. In: *The Economics and Management of Water and Drainage in Agriculture*, A. Dinar and D. Zilberman, eds. Kluwer, New York, pp. 169–186.

Ting, Y.-P., and G. Sun (2000) Use of polyvinyl alcohol as a cell immobilization matrix for copper biosorption by yeast cells. *J. Chem. Technol. Biotechnol.* **75**: 541–546.

Tobin, J.M. (2001) Fungal metal biosorption. In: *Fungi in Bioremediation*, G.M. Gadd, ed. Cambridge University Press, Cambridge, pp. 424–444.

Tobin, J.M., and J.C. Roux (1998) *Mucor* biosorbent for chromium removal from tanning effluent. *Water Res.* **32**: 1407–1416.

Tondeur, D., and G. Klein (1967) Multicomponent ion exchange in fixed beds: constant-separation-factor equilibrium. *Ind. Eng. Chem. Fundam.* **6**: 351–361.

Tsekova, K., D. Galabova, K. Todorova, and S. Ilieva (2002) Phosphatase activity and copper uptake during growth of *Aspergillus niger. Process Biochem.* **37**: 753–758.

Tsuruta, T. (2002) Removal and recovery of uranyl ion using various microorganisms. *J. Biosci. Bioeng.* **94**: 23–28.

Vasudevan, P., V. Padmavathy, and S.C. Dhingra (2003) Kinetics of biosorption of cadmium ion Baker's yeast. *Bioresour. Technol.* **89**: 281–287.

Volesky, B. (1994) Advances in biosorption of metals: selection of biomass types. *FEMS Microbiol. Rev.* **14**: 291–302.

Volesky, B. (2001) Detoxification of metal-bearing effluents: biosorption for the next century. *Hydrometallurgy* **59**: 203–216.

Volesky, B., and H.A. May-Phillips (1995) Biosorption of heavy metals by *Saccharomyces cerevisiae. Appl. Microbiol. Biotechnol.* **42**: 797–806.

Wasay, S.A., S.F. Barrington, and S. Tokunaga (1998) Using *Aspergillus niger* to bioremediate soils contaminated by heavy metals. *Bioremed. J.* **2**: 183–190.

Wilmoth, R.C., S.J. Hubbard, and J.O. Burckle (1991) Production and processing of metals: their disposal and future risks. In: *Metals and Their Compounds in the Environment: Occurrence, Analysis and Biological Relevance*, E. Merian, ed. VCH, Weinheim, Germany, pp. 19–65.

Woodward, B., and R. De Groot (1999) Tolerance of *Wolfiporia cocos* isolates to copper in agar media. *For. Prod. J.* **49**: 87–94.

Yalcinkaya, Y., L. Soysal, A. Denizli, M.Y. Arica, S. Bektas, and O. Genc (2002) Biosorption of cadmium from aquatic systems by carboxymethylcellulose and immobilized *Trametes versicolor. Hydrometallurgy* **63**: 31–40.

Yan, G., and T. Viraraghavan (2001) Heavy metal removal in a biosorption column by immobilized *M. rouxii* biomass. *Bioresour. Technol.* **78**: 243–249.

Yan, G., and T. Viraraghavan (2003) Heavy metal removal from aqueous solution by fungus *Mucor rouxii. Water Res.* **37**: 4486–4496.

Yannai, S., I. Berdicevsky, and L. Duek (1991) Transformations of inorganic mercury by *Candida albicans* and *Saccharomyces cerevisiae. Appl. Environ. Microbiol.* **57**: 245–247.

Yetis, U., G. Ozcengiz, F.B. Dilek, N. Ergen, A. Erbay, and A. Dolek (1998) Heavy metal biosorption by white-rot fungi. *Water Sci. Technol.* **38**: 323–330.

Yetis, U., A. Dolek, F.B. Dilek, and G. Ozcengiz (2000) The removal of Pb(II) by *Phanerochaete chrysosporium. Water Res.* **34**: 4090–4100.

Yin, P., Q. Yu, B. Jin, and Z. Ling (1999) Biosorption removal of cadmium from aqueous solution by using pretreated fungal biomass cultured from starch wastewater. *Water Res.* **33**: 1960–1963.

Zhang, L., L. Zhao, Y. Yu, and C. Chen (1998) Removal of lead from aqueous solution by non-living *Rhizopus nigricans. Water Res.* **32**: 1437–1444.

Zhang, D., J.A. Duine, and F. Kawai (2002) The extremely high Al resistance of *Penicillium janthinellum* F-13 is not caused by internal or external sequestration of Al. *BioMetals* **15**: 167–174.

Zhou, J.L. (1999) Zn biosorption by *Rhizopus arrhizus* and other fungi. *Appl. Microbiol. Biotechnol.* **51**: 686–693.

Zouboulis, A., E.G. Rousou, K.A. Matis, and I.C. Hancock (1999) Removal of toxic metals from aqueous mixtures. 1. Biosorption. *J. Chem. Technol. Biotechnol.* **74**: 429–436.

Zouboulis, A., K.A. Matis, M. Loukidou, and F. Sebesta (2003) Metal biosorption by PAN-immobilized fungal biomass in simulated wastewaters. *Colloids Surf. A* **212**: 185–195.

Zulfadhly, Z., M.D. Mashitah, and S. Bhatia (2001) Heavy metal removal in fixed-bed column by the macro fungus *Pycnosporus sanguineus*. *Environ. Pollut.* **112**: 463–470.

12

MYCORRHIZAL FUNGI IN RHIZOSPHERE REMEDIATION

12.1   INTRODUCTION

Mycorrhizas are symbiotic associations between certain soil fungi and plant roots and are ubiquitous in the natural environments. Their role in nutrient transport in ecosystems and protection of plants against environmental and cultural stress has long been known. The majority of the mycorrhizas are obligate symbionts because they have little or no ability for independent growth. Autobionts are not much involved in associations. By combining the structure and functional aspects of symbiosis, Trappe (1996) defined mycorrhizas as "dual organs of absorption formed when symbiotic fungi inhabit healthy organs of most terrestrial plants." Smith and Read (1997) defined a mycorrhiza as "a symbiosis in which an external mycelium of a fungus supplies soil-derived nutrients to a plant root." Due to research over the past five decades, several excellent books on mycorrhizas have been produced (e.g., Marks and Kozlowski, 1973; Harley and Smith, 1983; Allen, 1991; Smith and Read, 1997; Ramarao, 2002). Varma and Hock (1999) covered the mycorrhiza structure, function, molecular biology, and biotechnology. Read (1999) described the state of the art on mycorrhizas, including various definitions, contributions of mycorrhizas to the fitness of plants, enhanced pathogen resistance as a basis of increased fitness, and contribution of mycorrhizas in natural communities.

The vesicular–arbuscular mycorrhiza (VAM) is the most ancient type of association and colonizes the plants by scavenging for phosphate. About two-

thirds of plants are known to have a VAM type of association. The VAM association belongs to nearly 150 taxa of the Order Glomales. The second common type in the environmental systems are the ectomycorrhizal (ECM) fungi. The important differences between the ECM and VAM fungi have been summarized (Colpaert and Van Tichelen, 1996). The ECM fungi are more specialized in nutrient capture, and both fungi differ in the quantity of production of external biomass. These fungi are also called *extremophiles*, due to their occurrence in extreme habitats, including high or low temperature, pH, salt and metal concentration, drought, and so on. The growth of mycorrhizas is also known in the presence of low availability of nutrients that could increase plant growth.

The role of mycorrhizal fungi related to metal uptake and degradation of persistent organic compounds is emphasized in this chapter. The symbiotic relationship allows the fungus to tide over the harsh conditions of toxic contamination. The rhizosphere zone around the roots of plants is the active seat of metabolic substrates responsible for the growth and survival of fungi in soil ecosystems. The fungi and plant can extend nutrients to each other. However, the supply of nutrients in nutrient-deficient ecosystems by mycorrhizal fungi to the host plant is quite interesting. The role of mycorrhizas in rhizosphere remediation and of ECM fungi to degrade a wide variety of persistent organic compounds has finally been established (Meharg and Cairney, 2000a).

12.2   CLASSIFICATION OF MYCORRHIZAL FUNGI

Several different types of mycorrhizal associations have been classified from time to time by different mycologists. Certain species of fungi exhibit a narrow host range of plants, whereas others have a broad range. Worldwide distribution and a broad host range are known for the ECM fungus *Pisolithus tinctorius*. Some associations in forests are specific, whereas others are nonspecific. Some mycorrhizal associations influence the pattern of plant communities. The diverse communities of ECM, VAM, and ericoid mycorrhizal (ERM) fungi are known at many host plant root systems. The specificity of ECM fungi is well expressed. In a soil environment, levels of colonization depend on seasonal variations and activities related to soil disturbance. These variations lead to reduction in propagule density and mycelial systems.

Seven different types of associations have been classified: arbuscular, ectomycorrhiza, ectendomycorrhiza, ericoid, arbutoid, orchid, and monotrophic (Smith and Read, 1997). These categories are based on the criteria of the type of the fungus involved and the resulting structures produced by the root–fungus association. The host cells are not penetrated by ectotrophic mycorrhiza and form an intercellular hyphal network in a sheath around the plant roots. The host cells are penetrated by endomycorrhiza with a superficial hyphal network on the root surface. Read (1999) recognized six

categories based on the structural and major nutrient pathways. Such associations may not be mutualistic at all levels with respect to time. Manuals for the identification of mycorrhizal fungi (Ingleby et al., 1990; Schenck and Perez, 1990) have been published and a mycorrhizal manual (Varma, 1998) has been edited. Identification of VAM fungi is well epitomized in an article by Douds and Millner (1999). The anatomical characteristics of ectomycorrhizas (Agerer, 1999) and the morphological diversity among endomycorrhizal fungi in Glomales (Morton et al., 1999) related to systematics and natural classification have been described.

More than 6000 ECM fungal species (Molina et al., 1992; Bougher, 1994) and over 5400 species from 148 ECM genera (Allen et al., 1995) are known in the world. Ectomycorrhizal community structures have recently been described with reference to common taxa (Gehring et al., 1998; Jonsson et al., 1999a,b) and metabolism and gene expression (Wiemken and Boller, 2002). Olsson et al. (1996) demonstrated a reduction in or lack of bacterial activity near the mycelial network of ECM fungi. Bacterial populations can be inhibited as a result of nutrient competition by an extensive mycelium in the mycorrhizosphere (Green et al., 1999b).

12.3   FUNCTIONS OF MYCORRHIZAL MYCELIUM

One of the functions of the fungal mycelium associated with host plants is to enhance the volume of rhizosphere soil. Dominance of the mycelium in the upper soil layers is well known in temperate forest ecosystems, where it accounts for 45% of the total soil biomass. Undoubtedly, mycorrhizas have certain applications in agroforestry, soil stabilization, and land rehabilitation. Ectomycorrhizal fungi mobilize certain essential plant nutrients from rock by excreting a range of organic acids (van Breemen et al., 2000; Landeweert et al., 2001). This assists ectomycorrhizal plants to assimilate nutrients from insoluble sources of mineral and thus affects nutrient cycling in forests. However, in recent years, attention has been focused on the functions of the ectomycorrhizal mycelium in relation to transport.

Species of ECM fungi are similar to certain wood-rotting fungi in the pattern of mycelial growth and differentiation. The mycelium systems of mycorrhizal fungi are heterogeneous. The mycelium of ectomycorrhizal roots increases the efficiency of soil exploration in microcosms. In certain cases, mycelia are reported to exceed $300\,m^2$ (Bonello et al., 1998; Sawyer et al., 1999). Mycelial lengths of up to 2000 m/m root (Read and Boyd, 1986), 504 m/m (Rousseau et al., 1994), and 300 m/m (Jones et al., 1991) have also been noted. Smith and Read (1997) reported a mycelial surface area up to 47-fold. Hyphal lengths from 200 mg dry weight to 6.4 m in relation to soil weight have been recorded. Proliferation of fungal mycelia in the form of patches or mats in certain soils is also observed in microcosms and fields. This results in the capture of certain nutrients, such as nitrogen and phosphorus.

12.4 METHODS FOR STUDYING MYCORRHIZAL FUNGI

Several methods are known for the isolation of spores from the field soil for the study of ectomycorrhizal fungi: sieving and decanting (Vilarino and Arines, 1990; Douds and Millner, 1999), flotation–adhesion (Sutton and Barron, 1972), airstream fractionation (Tommerup, 1982), different water–sucrose centrifugation (Ianson and Allen, 1986), and fixing soil slurries to filter paper (Smith and Skipper, 1979; Khalil et al., 1994). A variation of the most probable number (MPN) method is used for the determination of colony-forming units (CFU) in fungi or bacteria (An et al., 1990). Trap culture techniques are applied for the detection of nonsporulating species. The quantification of VAM colonization of roots is through a dissecting microscope followed by staining with certain dyes. Quantification of fungal colonization is performed through microscopy or by measurement of fungus-specific compounds in the roots. Giovannetti and Mosse (1980) applied microscopy along with a gridline intersect method. The gridline intersect method has also been modified (McGonigle et al., 1990). Techniques for the study of mycorrhizal fungi (Norris et al., 1991, 1992) and a manual for basic techniques on the mycorrhizal research, including the establishment of micropropagated plants (Mukerji et al., 2002), have been discussed.

Several methods are known for the production of mycorrhizal inoculum and inoculum techniques (Kuek, 1994; Haselwandter and Bowen, 1996). Molina and Palmer (1982) described the isolation, maintenance, and pure culture of ECM fungi. A technique has been established to study ECM fungi on agar media (De Araujo and Roussos, 2002). *Suillus collinitus* and *Hebeloma cylindrosporum* have been grown on potato dextrose agar or Pintro's medium. The various methods have been compared to determine the metal sensitivity in ECM fungi in culture in the absence of a host and in symbiosis with a host in artificial substrates (Hartley et al., 1997). Protocols have been developed to study the effects of hydrocarbon pollution on VAM fungi (Cabello, 2001).

Fatty acids from abundant phospholipids (membrane structures) and neutral lipids (storage structures) of VAM fungi can be useful for estimating the biomass of infective VAM propagules. The biomass of the extraradical mycelium is 10-fold more than that of intraradical mycelium in VAM fungi (Olsson et al., 1999). Colpaert and Van Tichelen (1996) discussed many examples of the production of estimated biomass. The differences in fatty acid profiles are also beneficial in the identification of these fungi (Graham et al., 1995). Phospholipid fatty acid (PLFA) profiles can be coupled with radio- or stable-isotope analysis to distinguish the amount or presence of fungal or bacterial biomass (Green et al., 1999a). Fatty acid methyl ester (FAME) analysis was performed on the spores of four VAM fungi in a study by Madan et al. (2002). The FAME profiles confirm the use of $16:1\omega 5c$ as a marker fatty acid for VAM fungi in controlled environments and suggest

18:1ω9c, 20:1ω9c, 20:2ω6c, and 22:1ω9c as possible markers for the detection of *Gigaspora margarita*. The fatty acid 16:1ω5c was used to estimate the biomass of external mycelium of *Glomus geosporum* (Gaspar et al., 2002). Measuring the quantity of campesterol, 24α-methylcholesterol (Schmitz et al., 1991), ergosterol (Frey et al., 1994), and yellow carotenoid pigments in some mycorrhizal roots is also related to the extent of VAM colonization. Measurement of chitin is a common method for the determination of colonization of VAM fungi in roots. Chitin assays cannot distinguish between living and dead mycelia and provide variable results, due to differences in cell wall morphology.

Immunochemical methods are also known for the identification of these fungi. Different isolates can be identified with the production of monoclonal antibodies (Wright et al., 1987). The immunochemical properties of mycorrhizas are related to applications in taxonomy, host–symbiont dialogue, and ecological aspects (Hahn et al., 1999). A flowchart of specimen preparation for the study of ectomycorrhiza and optimal preparation pathways for cytochemical, immunocytochemical, microanalytical, and ultrastructural investigations have been discussed (Scheidegger and Brunner, 1999). Electron energy loss spectrometry (EELS), electron spectroscopic imaging (ESI), and energy-dispersive x-ray microanalysis (EDX) are widely used in mycorrhizal research.

Molecular methods such as polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) are also used in the identification of mycorrhizal fungi. A VANS1–NS21 primer pair with PCR has been used to detect VAM fungi colonizing roots of various plants (Simon et al., 1992; Di Bonito et al., 1995). These methods have gained success by amplifying nuclear DNA encoding for small-subunit rRNA and internal transcribed regions. A competitive PCR method has been developed in *Hebeloma cylindrosporum* that quantifies its DNA in complex mixtures extracted from soil (Guidot et al., 2002). The detection threshold of this protocol is 0.03 pg of genomic DNA. This method is applicable to belowground biomass underneath fruiting bodies and very close to them. A combination of morphotype and molecular technique using a primer, NL6B, has been employed to describe ectomycorrhizas (Goodman et al., 1996). Sequence-based markers to study the genetic structure and reproductive biology of *Suillus pungens*, including the basidiome genotypes, have been examined (Bonello et al., 1998). The genetic distribution and variation of *Cortinarius rotundisporus* by microsatellite-primed PCR (MS-PCR) and internal transcribed spacer (ITS) amplification and ITS-RFLP were studied by Sawyer et al. (1999). Kreuzinger et al. (1996) concluded that glyceraldehyde-3-phosphate dehydrogenase (*gpd*) genes could be used in the identification of ectomycorrhizal fungi under field conditions. Taxon-specific oligonucleotide probes have also been used to identify the fungal components of ectomycorrhizas (Bruns and Gardes, 1993). A combination of PCR and an oligonucleotide probe successfully identified

the diagnostic sequence from DNA preparations of both single spores and colonized roots (Yokoyama et al., 2002). Species of *Glomus* have been detected in cultivated soils using a PCR-denaturing gradient gel electrophoresis (DGGE) method for fungal 18S ribosomal RNA gene (Ma et al., 2005). Recently, the mycelium of the ectomycorrhizal fungi in soil has been quantified by real-time PCR (Landeweert et al., 2003).

12.5 MOLECULAR MECHANISMS OF MYCORRHIZAL SYMBIOSIS

At present, the molecular mechanisms of establishment of symbiotic mycorrhizas are not understood. A series of events are involved during interaction between the fungus and root cells, resulting in an integrated functional structure. This appears to be caused by activating and deactivating of genes in both fungus and host plant. Certain elicitors are produced by the root cells that regulate the expression of fungal genes to establish symbiosis (Burgess et al., 1995). Certain genes are activated, which are responsible for the development of a Hartig net and hyphal mantle (Salzer et al., 1997) and the deactivating of certain fungal gene encoding factors for host plant defense reactions. Certain elicitors present in ectomycorrhizal fungi are deactivated by chitinases of the root cortex without harming the fungus, thus establishing the formation of ectomycorrhizas (Salzer and Boller, 2000). The roots of host plants secrete signal molecule(s) that influence the growth of hyphae in VAM fungi in soil (Douds and Nagahashi, 2000). A dilute signal produces the hyphal branching pattern of *Gigaspora gigantea* in the form of long, scattered branches, and a concentrated signal causes tight clusters of profuse branching. This branching response is a mechanism suitable for host root colonization. Signaling mechanisms and molecular genetics of mycorrhizal symbiosis and genetic engineering of mycorrhizal fungi are described in a book edited by Podila and Douds (2000). A comparative analysis of molecular aspects of symbiosis, molecular regulation of nutrient exchange, and techniques of genetic transformation of viable transgenic mycorrhizal fungi have been described (Krishna, 2004). Stable genetic transformation and the expression of gene markers in transgenic *Pisolithus tinctorius* have recently been reported (Rodriguez-Tovar et al., 2005).

An in vitro *Laccaria bicolor* and *Pinus resinosa* interaction model system has been developed to identify and clone a symbiosis-regulated gene employing an mRNA differential display technique (DDRT-PCR) (Kim et al., 1998). The gene corresponding to PF6.2 is isolated and sequenced. Northern analysis reveals detection of PF6.2 mRNA in 6 hours after interaction and continues to be expressed in established ectomycorrhizas, suggesting its role in the formation and maintenance of symbiosis. This is the first report of cloning and characterization of a gene from ECM fungus involved in the initiation and maintenance of symbiosis.

12.6   METABOLISM OF MYCORRHIZAL FUNGI

12.6.1   General Metabolism

A supply of carbon by the host is necessary for the development of mycorrhizas. The establishment and maintenance of energy requirements for transport processes are important for both partners. A plethora of literature exists on the carbohydrate and energy metabolism of mycorrhizas. Nitrogen is also an essential nutrient for ectomycorrhizal fungi. Many researchers have described nitrogen assimilation, processes of uptake, and the physiology of organic nitrogen acquisition. Transfer cell structures within ECM fungi are also well recognized. Transport of nitrogen by ectomycorrhizal tissues, the soil–fungus interface, the fungus–apoplast interface, the apoplast–root cell interface, the quality of nitrogen compounds transferred, and the membrane transport has been featured by Chalot and Brun (1998), including a hypothetical model of transport systems for nitrogenous compounds in mycorrhizas.

12.6.2   Degradative Metabolism

The ECM fungi produce a variety of enzymes for the degradation of complex organic compounds in the soil. Mobilization of nitrogen from organic matter takes place by hydrolytic enzymes (proteinases and peptidases) in certain ericoid and ectomycorrhizal fungi. Extracellular proteinase activities are detected in ericoid and ectomycorrhizal fungi on protein as a major growth substrate. Exocellular proteinase activities can be produced and are characterized in ericoid and ectomycorrhizal fungi. Several ECM fungi also produce amylase, gelatinase, lipase, and urease (Hutchinson, 1990). Certain mat-forming ectomycorrhizal fungi show elevated levels of cellulase, phosphatase, peroxidase, proteinase, and so on, compared to nonmat types.

Several VAM fungi produce xylanases, mannases, and other glycanase complexes that may aid in the partial degradation of hazardous compounds. Cellulolytic enzyme activities in ECM fungi have also been detected (Maijala et al., 1991). Cairney and Burke (1996) reported the expression of extracellular endoxylanase activity by four isolates in both ECM and ERM fungi, and the highest activity was recorded in *Hymenoscyphus ericae*. These enzymes may play a role at the fungus–root interface and in extramatrical mycelium in ECM and ERM fungi. Chitinases play a role in morphogenesis with multiple functions. The current status of hydrolytic enzymes (e.g., cellulases, hemicellulases, pectinases, and chitinases) from VAM fungi has been described (Varma, 1999).

Some ECM and ERM fungi contribute a wide range of phenol-oxidizing enzymes, such as tyrosinase, catechol oxidase, ascorbate oxidase, and laccase (Bending and Read, 1995; Colpaert and Van Laere, 1996; Gramss, 1997; Timonen and Sen, 1998; Burke and Cairney, 2002). The activities of non-

specific phenol oxidase enzymes in axenic culture and symbiosis are known in ECM fungi (Bending and Read, 1996a; Gunther et al., 1998; Gramss et al., 1999). Exolaccase production by *Thelephora terrestris* (Kanunfre and Zancan, 1998) is known to catalyze oxidation of phenols to phenoxy radicals. The oxidases can be responsible for the transformation of PAHs, but this has not been confirmed.

Bavendamm test is employed for the detection of extracellular oxidases and agar spot tests for tyrosinase, laccase, and peroxidase enzymes in ECM and in wood- and litter-decaying fungi (Gramss et al., 1998). Remarkable extracellular oxidase activities have been detected in species of *Lactarius* and *Russula*. Tyrosinase has been detected in all isolates. The spot test color is restricted to the mycelium, suggesting an intracellular enzyme in species of *Amanita*, *Hebeloma*, *Leccinum*, *Suillus*, and *Tricholoma*. A strong extracellular laccase reaction has also been observed. However, intracellular laccase dominates in some ECM fungi. All isolates show polyphenol oxidase as the catecholase/monophenol monoxygenase complex despite difficult detection in the presence of laccase. Burke and Cairney (2002) summarized assay and substrate of polyphenol oxidase activities, including laccase from tissues of ERM and ECM fungi. Polyphenol activity in the taxonomy of ECM fungi in axenic culture has been proposed (Hutchinson, 1990). Four groups have been identified based on the detection or nondetection of laccase and tyrosinase. Liquid culture mycelia of *Suillus granulatus* produced intracellular activities in tyrosinase, laccase, and peroxidase (Gunther et al., 1998). Extracellular laccase and tyrosinase activities are also found in the culture fluid. *Paxillus involutus* produces mainly intracellular laccase. Tyrosinase activity is found in *Suillus granulatus-Pinus sylvestris* symbiosis. The release of these enzymes can contribute humification and detoxification processes in soil.

Several studies indicate the partial mineralization of lignin by both ERM and ECM fungi. This appears to be indicative of the production of peroxidative enzymes (i.e., lignin peroxidase and manganese peroxidase) (Griffiths and Caldwell, 1992; Cairney and Burke, 1994). Cairney and Burke (1998) attested that no evidence existed to support the production of peroxidase enzymes by ERM and ECM fungi but that the enzymes could be expressed under certain circumstances. The detection of low lignocellulase activity in both mycorrhizal fungi (Colpaert and Van Laere, 1996) indicates a limited capacity to decompose the lignocellulose components. The same seems true of such a low activity in the field conditions. Isolates of the ERM fungus *Hymenoscyphus ericae* and the ECM fungi *Suillus variegatus* and *Pisolithus tinctorius* are capable of producing extracellular H_2O_2 during axenic growth on modified Melin–Norkrans agar medium (MMN) containing either glucose or cellobiose as a carbon source (Burke and Cairney, 1998). Cellobiohydrolase, β-D-glucosidase, cellobiose oxidase, and glucose oxidase activities are expressed on glucose- and cellobiose-containing media, respectively. Cellobiose oxidase appears to be localized in the periplasm. This was already known in wood-degrading fungi, where it excretes H_2O_2 from hyphae, and

this is a first step in lignin degradation by saprophytic fungi. The mycelial extracts of all isolates generate the hydroxyl radical (·OH) in the presence of cellobiose and Fe(III), mediated by H_2O_2 produced by cellobiose oxidase activity. Chambers et al. (1999) demonstrated MnP activity in *Tylospora fibrillosa* and a gene equivalent to a known isozyme from *Phanerochaete chrysosporium*. Recent data support the identification of genes of lignin peroxidase and to a lesser extent, manganese peroxidase in a broad taxonomic range of ECM fungi (Chen et al., 2001). It appears that these enzymes are induced under certain conditions in some taxa.

12.7 UPTAKE OF TOXIC METALS

Plants that grow on heavily polluted soils are known as mycotrophic. ECM, ERM, and VAM fungi can increase plant tolerance to heavy metals at toxic concentrations. This is due to the accumulation of metals in extramatrical hyphae and extrahyphal slime. This leads to the immobilization of metals in or near roots and decreases uptake to shoots. Increased levels of metal tolerance to plants are not known in all mycorrhizal associations, due to differences in influence on plant metal tolerance. ECM fungi may be useful as bioindicators of pollution (Haselwandter et al., 1988; Aruguete et al., 1998).

Mine spoils are good target sites from which to study the application of mycorrhizal fungi. The influence of soil acidification is generally difficult to separate from the influence of metals. Many ECM fungi are associated with acidic, nutrient-poor soils in coniferous forests, and these acidic soils increase the solubility of Al and Mn ions (Dighton and Jansen, 1991). Species of ECM fungi exhibit constitutive tolerance to acidic conditions and high metal concentrations. Inoculation with *Pisolithus tinctorius* at the root of *Pinus massoniana* increases its ability to resist toxicity due to Al stress (Kong et al., 2000).

12.7.1 Metal Tolerance in Mycorrhizal Symbiosis

ECM fungi are metal sensitive in pure solid and liquid cultures. Based on soil concentration*, Pinus* and *Pices* seedlings can be protected from heavy metal toxicity by *Suillus luteus* (Dixon and Buschena, 1988). VAM fungi decrease the concentrations of Cd, Mn, and Zn in leaves of host plants in highly metal-polluted soils (Heggo et al., 1990; Hetrick et al., 1994). A metal tolerance of ECM fungi in symbiosis with a host plant is noted in Table 12.1. Several studies are known for in vitro tolerance of ECM fungi against heavy metals. Perhaps no correlation exists between in vitro tolerance of fungi and their expression of increased metal tolerance of plants. A combination of in vitro, in vivo, and field studies are important for the evaluation of metal tolerance of ECM fungi. The percentage of mycorrhizal root tips can be related to the

TABLE 12.1   Metal Tolerance of Mycorrhizal Fungi in Symbiosis

Mycorrhizal Fungus	Host	Biotest Type	Growth Substrate	Concentration Range of Metal(s)	Duration (days/weeks/ months)	Reference
Pisolithus tinctorius	*Eucalyptus urophylla*	In vitro	MMN medium with glucose and malt extract	0–1000 μM Cr/l 0–160 μM Ni/l	10–60d	Aggangan et al., 1998
		In vivo	Oven-dried pasteurized yellow sand	0–60mM Ni/kg	12w	
VAM fungus	*Trifolium repens*	Greenhouse	Sterile soil/sand mixture	0–400mg Zn/kg	20w	Zhu et al., 2001
Glomus mosseae	*Trifolium pratense*	Greenhouse	Sterile pasture soil	0–1000mg Zn/kg	62d	Li and Christie, 2001
Suillus bovinus *Paxillus involutus* *Thelephora terrestris*	*Pinus sylvestris*	Greenhouse	Vermiculite	900mg Zn/l	3m	Colpaert and Van Assche, 1992
Suillus bovinus *Paxillus involutus* *Thelephora terrestris*	*Pinus sylvestris*	Greenhouse	Perlite	44.5 μM Cd	6m	Colpaert and Van Assche, 1993
Laccaria laccata *Paxillus involutus* *Pisolithus tinctorius*	*Picea abies*	Greenhouse	Acid-washed quartz sand	5.0 μM Pb	32 or 42d	Marschner et al., 1996
Acaulospora laevis *Glomus caledonium* *Glomus manihotis*	*Zea mays*	Greenhouse	Acid-washed quartz sand	0.05, 0.2, 1, 3mg/l Cu 0.05, 0.1, 0.5, and 1mg/l Cd	8w	Liao et al., 2003

measurement of metal tolerance in vitro and in vivo. Heavy metal tolerance under in vivo conditions indicates a potential for future inoculations of *Eucalyptus* on ultramafic soils (Aggangan et al., 1998).

Mycorrhizas of *Cortinarius semisanguinea* exhibit the highest mean levels of Cu and Zn, while those of *Russula* spp. and *Suillus* spp. display the highest mean levels of Pb and Cd, respectively (Berthelsen et al., 1995). These authors also discuss the various morphological types. Levels of Zn (~38%), Cd (~33%), and Pb (~2%), respectively, were accumulated in the fungal biomass. Increasing the Pb concentration produced different ECM morphotypes in soil (Chappelka et al., 1991). In glasshouse investigations, five ECM morphotypes were identified in association with seedlings of *Pinus sylvestris* (Hartley, 1997). However, the addition of Cd > 20 mg/kg soil or Zn > 200 mg/kg soil revealed only two morphotypes.

Smith and Read (1997) propounded several different mechanisms for the interactions between colonization of mycorrhizas and accumulation of heavy metals, including interactions with phosphorus nutrition leading to tissue dilution of the toxic element, metal sequestration, and tolerance development by the fungus. However, the results presented by Zhu et al. (2001) cannot be explained according to their mechanisms. Brooks (1998) reported increased transport of metal to the shoots by hyperaccumulator plant species that developed tolerance to high metal concentrations. Additional studies are needed on mycorrhiza hyperaccumulators as a mechanism of protection against heavy metals in the soil.

The protective effect of mycorrhiza against plant Zn uptake has been observed (Li and Christie, 2001). This correlates with changes in Zn solubility due to changes in soil solution pH or by immobilization of Zn in extraradical mycelium. Cairney and Meharg (1999) reported that the diversity of ECM fungi and infections could be maintained in multiple contaminated sites. In this way, fungus provides protection to a plant against contaminants and assists the plant to grow in hazardous areas. During sand culture, *Glomus caledonium* shows the highest infection rates and the poorest sporulating ability in heavy metal (Cu and Cd) treatments (Liao et al., 2003).

12.7.2 Mechanisms of Response to Metals

The mechanisms of the mycorrhizal fungi against metal toxicity and host plants and to them are not well understood. However, several mechanisms have been postulated. Many of the principles are similar to the biosorption of other classes of fungi. The mechanisms can be divided into two categories: avoidance and sequestration. Avoidance can reduce the concentration of metal by precipitation, biosorption, and uptake or efflux. Sequestration involves the formation of compounds for intracellular chelation, and so on. The binding of heavy metals on extraradical mycelium is suggested as the mechanism for the tolerance of heavy metals in ericacious mycorrhizal and ectomycorrhizal plants. Abundant production of extramatrical mycelium can

provide the best protection to the host, as it provides increased capacity for metal retention by reducing exposure to the individual hyphae. X-ray micro-analysis has located high Zn concentrations in extramatrical hyphae in cell walls and extrahyphal polysaccharide slime (Denny and Wilkins, 1987). The same is also true in ericoid mycorrhizal endophytes (Denny and Ridge, 1995). Dense mycelium can also provide an increased supply of nutrients to the host and will infect new plant roots due to the larger biomass. In certain mycorrhizal associations, the fungal sheath can prevent metals from reaching the root surface. In general, these fungi produce loosely packed sheaths with large interhyphal spaces. The permeability of the apoplastic pathway to solute movement of the fungal sheath can play an important role in determining fungus-mediated host metal tolerance. The high metal storage capacity of *Xerocomus badius–Picea abies* in acidic soil is related to both the activity of the hyphal sheath and the frequent occurrence of vacuoles (Kottke et al., 1998).

In ECM fungi, polyphosphates are produced in the vacuole that exist in the form of insoluble granules complexed with a variety of cations. Polyphosphate granules bind the metal cations, and this appears to be a method of intracellular metal detoxification in these fungi. Electron spectroscopic imaging and electron energy loss spectroscopy reveal two types of electron-opaque granules detected in vacuoles of Harting net hyphae in *Paxillus involutus-Pinus sylvestris* mycorrhizas of heavily polluted sites (Turnau et al., 1993, 1996). One type of granule detects high concentrations of P with S, Ca, and Al, and the other type, low concentration of P with more N, S, and Cd. Polyphosphate granules are localized inside the cytoplasm of the fungal mantle and Hartig net and contain P and Ca (Grellier et al., 1989; Moore et al., 1989). The ability of *Paxillus involutus* to accumulate Cd in different compartments has been recognized (Blaudez et al., 2000). Two mechanisms of metal detoxification can be considered: binding of Cd onto cell walls and accumulation of Cd in the vacuolar compartment. *Oidiodendron maius* Cd 8-*Vaccinium myrtillus* mycorrhizas of polluted soils display the formation of insoluble Zn crystals (Martino et al., 2003). Isolates of *O. maius* produce citric, malic, and fumaric acids that solubilize the insoluble Zn compounds. Strains isolated from unpolluted soils are more efficient in solubilization from both ZnO and $Zn_3(PO_4)_2$ than strains from polluted soils. Increased tolerance to arsenate has been found for *Hymenoscyphus ericae* collected from polluted soils (Sharples et al., 2001). Arsenic enters the cell via the phosphate transporter. A higher tolerance of Zn and Cd of *Suillus luteus* isolates from a polluted habitat is expressed in vitro than of isolates from an unpolluted habitat (Colpaert et al., 2000). Nearly 70 to 90% of ERM fungi solubilize Cd and Cu phosphates and cuprite (Fomina et al., 2005). Also, toxic metal minerals are better solubilized by metal-tolerant isolates.

Other metal chelators are found in the vacuoles of fungi: inorganic ions, phytochelatins, metallothioneins, and organic acids. Chelators may vary from

ECM species to species and nutrient status of the fungus. Different metals also have varying affinities for chelators. Zn and Al show higher affinity for organic acids and Cu and Cd for phytochelatins. Metallothionein-like peptides are induced in *Pisolithus tinctorius* in the presence of Cu, Cd, and Zn (Morselt et al., 1986). Cd exposure leads to increased activity of adenosine 3′-phosphate 5′-phosphosulfate sulfotransferase, sulfate reduction, and acid-soluble thiols in *Laccaria laccata* in symbiosis with *Picea abies* (Galli et al., 1993). Forming complexes with glutathione and γ-glutamylcysteine detoxifies Cd. Polyamine formation in response to metals is also known. Exposure of Cu to ECM fungi propels an increase in activity of extra- and intracellular tyrosinase (Gruhn and Miller, 1991). The activity of tyrosinase enhances melanin, which limits the entry of Cu and other ions into the cells. On the contrary, little polyamine formation is noticed in *Paxillus involutus* due to Zn exposure (Zarb and Walters, 1995). Metallothioneins and phytochelatins are involved in constitutive tolerance (Meharg, 1994). The plasmalemma of ECM fungi also appears to protect the host plant from metal toxicity. Metallothioneins are isolated and characterized from the ECM fungi *L. laccata* and *Paxillus involutus* (Howe et al., 1997). Some open reading frames encoding for putative metallothioneins have been identified on the chromosomes of the VAM fungus *Gigaspora margarita* (Lanfranco et al., 2002) and the ECM fungus *Tuber borchii* (Pierleoni et al., 2004).

VAM fungi have also been recognized on hosts colonizing metal-contaminated soils (Meharg and Cairney, 2000b). It appears that VAM fungi have evolved resistance to act as the nutrient supply or provide increased resistance to host plants. VAM fungi from arsenic mine spoils also contribute to plant tolerance (Gonzalez-Chavez, 2000). This study indicated an increase in the arsenate resistance of the arsenate-resistant grass *Holcus lanatus*.

12.7.3   Transport of Radionuclides

Fungi can determine the fate and transport processes of radionuclides in forests (Steiner et al., 2002). They play an important role in uptake, mobilization, and translocation of radionuclides and are responsible for the long-term retention of radiocesium in organic horizons of forest soil. Due to limited knowledge on mechanisms and processes, an attractive approach utilizing transfer factors and concentration ratios is employed to quantify the transfer of radionuclides from soil to fungal fruiting bodies. Transfer factors are expressed as parameters of contamination of fungal fruiting bodies divided by parameters of soil contamination. Different definitions of transfer factors have been developed for the radioecological models with different purposes. Transfer factors measure the availability of uptake of radionuclides directly and reflect the appropriate physicochemical and biological processes. Transfer factors for radiocesium and stable cesium for three fungal species and *Vaccinium myrtillus* in a seminatural coniferous forest have been calculated (Ruhm et al., 1999). The relationship between radiocesium and stable cesium

in mushrooms from forest ecosystems with different contamination levels has been summarized (Yoshida et al., 2000).

A few processes related to the persistence of radiocesium in the upper layers of forest soil have been identified. Fungal activity in the forest soil can be considered in the context of predictive modeling. Quantitative prediction of radiocesium concentrations in different horizons can be conducted using compartment models (Ruhm et al., 1996; Schell et al., 1996). The applicability of compartment models for simulating the transport of radionuclides in soil has been discussed (Kirchner, 1998). A three-phase model to quantify radiocesium migration in soils of coniferous forest has been proposed (Rafferty et al., 2000).

Organic horizons of soil in forest ecosystem contain a major contamination of radiocesium from Chernobyl fallout (Rafferty et al., 1997; Linkov and Schell, 1999). Radiocesium is still accumulated in surface layers in Japanese forests 50 years after the global fallout (Yoshida and Muramatsu, 1994). About 22% of ^{137}Cs bound by fungal mycelia is estimated to be in the top 5 cm of a coniferous forest (Fawaris and Johanson, 1995). A major fraction of ^{137}Cs in a Swedish forest seems to be associated with the fungal component (Nikolova et al., 2000). Only 0.03% of the total ^{137}Cs is found in the mycelium of ectomycorrhizal fungi in the peat soil (Vinichuk et al., 2004). The value is higher for the nonpeat soil. Horizontal transport of radiocesium is known in Scandinavian forests in the form of local enrichment by fungal clusters (Nikolova et al., 1997). Autoradiography followed by image analysis reveals the translocation of radiocesium by bulk flow in actively expanding mycelia of *Schizophyllum commune* in microcosms and by diffusion in established mycelia under laboratory conditions (Gray et al., 1995).

Plants inoculated with a specific mycorrhizal fungus have increased their ability to obtain necessary nutrients while removing large quantities of ^{137}Cs and ^{90}Sr from contaminated soils (Rogers and Williams, 1986; Entry et al., 1994). However, the mycorrhizal heather *Calluna vulgaris* accumulates less ^{137}Cs in liquid medium than do nonmycorrhizal plants (Clint and Dighton, 1992). Three grass species inoculated with *Glomus mosseae* or *G. intraradices* remove 26 to 72% of ^{137}Cs and 24 to 89% of ^{90}Sr from contaminated soil (Entry et al., 1999). Grasses inoculated with these fungi exhibit higher aboveground plant biomass, higher concentration of ^{137}Cs and ^{90}Sr in plant tissue, percentage accumulation of ^{137}Cs and ^{90}Sr from soil, and plant bioconcentration ratios at each harvest. Inoculation of grasses with VAM fungi offers a feasible, low-cost strategy to remediate and reclaim some sites contaminated with radionuclides. *Hebeloma crustuliniforme* reduces the concentrations of ^{134}Cs in *Picea abies* seedlings from solutions of CsCl and KH$_2$PO$_4$ with different Cs/K ratios (Brunner et al., 1996). VAM fungi related to heavy metal uptake and radionuclides from soil have also been discussed (Haselwandter et al., 1994). The heavy metals and lanthanides concentrations in sporocarps of U.S. ectomycorrhizal fungi and factors affecting metal uptake in ectomycorrhizal fungi have been documented (Aruguete et al., 1998).

12.7.4 Genetics of Metal Tolerance

No studies have been conducted on the genetics and molecular biology of ECM fungal adaptation to contaminated soils in the fields. Such studies are needed to investigate the mechanisms of ECM metal tolerance. Such studies may include the screening of sensitive and insensitive genotypes from mine and wild-type ECM fungal populations. Segregation of metal-sensitive and metal-insensitive isolates on metal-contaminated soils can lead to isolation of adaptive metal-tolerant species. Physiological studies of metal-tolerant species can reveal the mechanism of metal adaptation. Tomsett (1993) described some aspects of genetics and molecular biology of metal tolerance in fungi. Isolates of *Pisolithus tinctorius* from a contaminated acid coal mine site show varied tolerance to Al, with EC_{50} values from 0.2 to 74 mM, compared to 0.04 to 0.07 mM Al tolerance for wild-type isolates from a contaminated site (Egerton-Warburton and Griffin, 1995). The genetic variation in the population of *Suillus luteus* is larger than at the polluted site (Colpaert et al., 2000). The 12 *Oidiodendron maius* isolates from the polluted soils were grouped in different genetic clusters by Lacourt et al. (2000).

Glomalin, an insoluble glycoprotein produced by VAM fungi can sequester potentially toxic elements such as Cu, Cd, Pb, and Mn (Gonzalez-Chavez et al., 2004). Glomalin extracted from polluted soils contained Cu, Cd, and Pb. Glomalin from hyphae of an isolate of *Gigaspora rosea* sequestered up to 28 mg Cu/g in vitro. Glomalin can act as a biostabilizer for the remediation of polluted soils.

12.8 PETROLEUM HYDROCARBON DEGRADATION

Crude oil spills are global problems and cause severe damage if released into the environment. The toxic effects of crude oil on plants are established, but little is known on the oil degradation by mycorrhizal fungi. Miller et al. (1978) described a significant reduction in the infectivity of ECM and its biomass in the aerobic microflora of Arctic tundra, and the resistance to oil is noted in certain fungi. Rajapakse and Miller (1991) discussed methods for the evaluation of ectomycorrrhizal tips and VAM colonization in poplar roots. The petroleum hydrocarbon-degrading capabilities of mycorrhizal fungi are limited and are noted in Table 12.2.

ECM fungi exhibit extensive colonization of spruce plants sown at the time of contamination, and most spruce root tips (90%) are mycorrhizal (Nicolotti and Egli, 1998). Six morphotypes have been identified and their macroscopic and microscopic features noted. Type E displays the best colonization, matching exactly the growth pattern of three species of *Laccaria* in pure culture and morphological characteristics, suggesting that type E may belong to this genus. VAM fungi occur in both petroleum-polluted and nonpolluted soils (Cabello, 1997). Nonpolluted soils display higher percentages of colonization

TABLE 12.2 Petroleum Hydrocarbon-Degrading Capabilities of Mycorrhizal Fungi

Mycorrhizal Fungus	Host	Biotest Type	Duration (days/weeks/months)	Oil/Medium	Reference
VAM fungi					
Glomus aggregatum	*Cynodon dactylon*	Greenhouse	8w	Petroleum-polluted soils	Cabello, 1997
Glomus mosseae	*Solidago* sp.				
	Dactylis glomerata			Nonpolluted soils	
	Cynodon dactylon				
	Bromus brevis				
	Melilotus sp.				
ECM fungi					
Amanita pantherina	*Populus nigra*	In vitro and greenhouse	35 d	Crude oil	Nicolotti and Egli, 1998
Cenococcum geophilum	*Picea abies*				
Hebeloma crustuliniforme			4 m		
Tricholoma vaccinum					
Pisolithus tinctorius					
Laccaria bicolor					
Laccaria amethystea					
Laccaria laccata					
Paxillus involutus or	*Pinus sylvestris*	Test-tube microcosm	16 w	Petroleum-polluted soils	Sarand et al., 1998
Suillus bovinus					
Suillus bovinus and	*Pinus sylvestris*	Test-tube microcosm	25 d	*m*-Toluate	Sarand et al., 1999
Pseudomonas fluorescens					

in plants than do polluted soils. The proportion of arbuscles to vesicles and number of entry points are higher in plants grown in uncontaminated soils. The vesicles show signs of degeneration due to the utilization of lipid reserves by the fungus. The highest propagule densities per 100 g of dry soil to colonize roots are found in uncontaminated soils. VAM infective propagules from contaminated soils display the high colonization capacity. The VAM fungal species *Glomus aggregatum* and *G. mosseae* have been identified from both contaminated and uncontaminated soils.

Several reports have documented an influence on ECM symbiosis by anthropogenic pollution from different industrial and/or urban sources; however, it is difficult to correlate such results. Cairney and Meharg (1999) discussed the influence of important forms of anthropogenic pollution on the morphology and dynamics of mycorrhizal fungal communities. Change in infection levels and diversity in ECM morphotype have been reported as an outcome of these studies. Knowledge of changes in mycelial systems by such pollution will provide an understanding of the sustainability of mycorrhizal systems. A range of soil pollutant parameters present in industrial and/or urban sources have been measured (Markkola et al., 1995; Kieliszewska-Rokicka et al., 1997).

It is evident that the long-term environmental consequences of oil spills in agricultural and forest areas are not of much immensity compared with coastal ecosystems. This may be due to the presence of fungi and bacteria in the soil that can catalyze the process of bioremediation. Certain ectomycorrhizal fungi can survive in these contaminated soils, where these can utilize crude oil as a nutrient and contribute their share in the process of mycoremediation. Mycorrhizal fungi can also establish degradative consortia in the root zone. An example of such a mycorrhizal system results in the formation of a parenchymatous patch on hydrocarbon-contaminated soil supporting a hydrocarbon-degrading bacterial biofilm (Sarand et al., 1998). When inoculated with a strain of *Pseudomonas fluorescens* containing a toluene-degrading plasmid, the same rhizosphere association reveals the degradation of *meta*-toluate (Sarand et al., 1999). Besides, more knowledge is required on the contribution of mycorrhizal fungi in petroleum hydrocarbons, tolerance limits, role of nutrients, host growth and survival, and the various infective propagules in the soils.

12.9 LIGNIN AND PHENOLIC DEGRADATION

Phenolics are rich in the soils of healthland and temperate-forest ecosystems. Plants associating with ECM and ERM fungi inhabit soils containing phenolic compounds. Two broad categories are known for these compounds: lignin and soluble phenolics, including phenolic acids and polyphenols (e.g., tannins, quinones, and humic and fulvic acids). Polyphenols can bind with a range of

organic nitrogen compounds, such as proteins, nucleic acids, and chitin, resulting in the formation of complexes.

A limited number of ECM and ERM fungi have shown the ability to degrade lignin and soluble phenolics (Table 12.3). Lignin and phenolics can be degraded in axenic cultures. In pure cultures, five different species of ECM fungi show their ability to metabolize ^{14}C-labeled plant lignin, [^{14}C]lignocellulose, and [^{14}C]DHP–lignin at a rate lower than the white-rot fungi *Heterobasidion annosum* and *Sporotrichum pulverulentum* (Trojanowski et al., 1984). ECM fungi exhibit relatively less degradation of [^{14}C](U)-holocellulose than that of white-rot fungi. Two species of ECM fungi decompose [^{14}C]vanillic acid to a higher extent than *Heterobasidion annosum*. The ERM fungi are more effective in degrading [^{14}C]lignin than ECM fungi in pure cultures (Haselwandter et al., 1990). The facultative ECM fungus *Paxillus involutus* degrades lignin more readily than the obligate ECM fungi *Suillus bovinus* and *Rhizopogon roseolus*. ERM fungi and ECM fungus *Paxillus involutus* release more than 35% and 24% of [[14]CH$_3$]DHP of coniferyl alcohol, respectively.

ERM fungi can detoxify phenolics (Bending and Read, 1996a), whereas ECM and ERM fungi bind tannin–protein complexes through nitrogen (Bending and Read, 1996b). The ERM fungus *Hymenoscyphus ericae* readily metabolized all but benzoic and *para*-methoxybenzoic acids in axenic culture (Leake, 1987). Vanillic, *para*-coumaric, and *para*-hydroxybenzoic acids increased the mycelial yields, while ferulic, syringic, and salicylic acids and an equimolar mixture of compounds retarded it. A carbon source and concentration showed no effect on the degradation of *para*-hydroxybenzoic acid or catechol by *Armillaria ostoyae* (Entry et al., 1992). *A. ostoyae* grows faster at high carbon concentration, but cannot more effectively degrade *para*-hydroxybenzoic acid or catechol. The phenol-degrading abilities of ERM fungi are more developed than the limited abilities of ECM fungi. Phenol-oxidizing enzymes are responsible for the degradation of phenols by these fungi.

Suillus granulatus completely metabolizes catechol, 3,4-dihydroxybenzoic acid, and vanillic acid, which have been attributed to tyrosinase activity (Gunther et al., 1998). However, *Paxillus involutus* metabolized these compounds at low rates. Mycelia of *S. granulatus* led to complete transformation of *para*-cresol within 5 hours. Two metabolites similar to 4-methylcatechol and 4-methyl-*ortho*-benzoquinone were produced after 1 hour. Cell-free culture fluid of *S. granulatus* oxidized *para*-cresol at a lower rate (i.e., 50% after 30 hours, with the formation of minor concentrations of the same two metabolites). Mycelia of *P. involutus* oxidized *para*-cresol at a low rate without the formation of metabolites, and the cell-free culture fluid showed no activity. *Suillus granulatus–Pinus sylvestris* mycorrhizas indicated the disappearance of *para*-cresol within 48 hours with the detection of same two metabolites. *P. involutus* mycorrhizas resulted in a 10% reduction of *para*-cresol initial concentration without the detection of metabolites.

TABLE 12.3 Lignin and Phenolic-Degrading Capabilities of Mycorrhizal Fungi

Mycorrhizal Fungus	Host	Biotest Type	Constituent	Duration (days)	Reference
ECM fungi					
Cenococcum geophilum		Pure culture	[14C] plant lignin	30	Trojanowski et al., 1984
Amanita muscaria			[14C] lignocellulose		
Tricholoma aurantium			[14C] DHP lignin		
Rhizopogon luteolus					
Rhizopogon roseolus					
ECM fungi					
Paxillus involutus		Pure culture	[14C] lignin	20–60	Haselwandter et al., 1990
Suillus bovinus			[O14CH3] DHP of		
Rhizopogon roseolus			coniferyl alcohol		
ERM fungi					
Hymenoscyphus ericae					
Oidiodendron griseum					
Hymenoscyphus ericae	Calluna vulgaris Festuca ovina	Pure culture	Benzoic and p-methoxybenzoic acids	56	Leake, 1987
Hymenoscyphus ericae		Pure culture	Phenolic acids and tannins		Leake and Read, 1991
Hymenoscyphus ericae		Pure culture	Tannic acid	7	Bending and Read, 1996a
ECM fungi					
Paxillus involutus		Axenic culture	Benzoic	28	Dittmann et al., 2002
Suillus bovinus			4-hydroxybenzoic acid		
Suillus bovinus	Pinus sylvestris		3-Chlorobenzoic acid	28	
Paxillus involutus		Axenic culture	2,4-Dichlorophenol	17	Meharg et al., 1997a
Suillus variegatus	Pinus sylvestris		2,4-Dichlorophenol	13	
Suillus granulatus		Axenic culture	Catechol	3	Gunther et al., 1998
Paxillus involutus			3,4-Dihydroxybenzoic acid		
			Vanillic acid		
			Ferulic acid		
			p-Cresol		
	Pinus sylvestris		p-Cresol	2	

The tannin-degrading abilities of certain mycorrhizal fungi have been identified (Giltrap, 1982; Bending and Read, 1996a). ECM fungi are recognized for lower-degrading abilities of tannic acid (Trojanowski et al., 1984; Haselwandter et al., 1990). Ericoid *Hymenoscyphus ericae* shows well-developed abilities to degrade tannin (Bending and Read, 1996a). The inhibition of growth of fungi by tannic acid may be due to the inactivation of enzymes of the cell wall. Degradation of tannic acid is enhanced in the presence of assimilation of NH_4^+. Protein binding capacity is reduced significantly as a result of tannic acid degradation by *H. ericae*. *H. ericae* induces the production of extracellular polyphenol oxidase in the presence of tannic acid, which is not the case with ECM fungi. Addition of NH_4^+ seems to enhance the synthesis of phenol-oxidizing enzymes, which in turn increases both the mycelial yields and tannic acid degradation by *H. ericae*. This process may be accelerated in certain healthland soils that contain small amounts of NH_4^+.

A vermiculite culture system inoculated with fungi in the presence or absence of a host has also been employed for the assay of mineralization of 2,4-dichlorophenol (Meharg et al., 1997a). This is a first report on the demonstration of the ability of ECM fungi related to the degradation of aromatics in axenic cultures and by intact mycorrhizal associations. Higher mineralization occurs during growth of fungi in symbiosis with *Pinus sylvestris* than in the absence of a host. *Suillus variegatus* reveals a higher growth rate of phytobiont, with seedlings of shoot biomass of 230 mg as compared to a *P. involutus* biomass of 150 mg. Both roots and shoots exhibit the accumulation of label 2,4-dichlorophenol, about 40% of the total plant biomass by two fungi. *Paxillus involutus* stimulates more efficiently than *S. variegatus* in labeling 2,4-dichlorophenol during vermiculite culture studies in the absence of seedlings. The pattern of stimulation is significantly different in both fungi in symbiotic association with the Scots pine plant. *P. involutus* increases 50% mineralization and *S. variegatus* 250% in symbiotic association. Only 3% of 2,4-dichlorophenol is mineralized after 13 days in vermiculite investigations by the most efficient degrader. In one study, 2,4-dichlorophenol was metabolized rapidly in both rhizosphere and nonrhizosphere soils, and 25% of 2,4-dichlorophenol was mineralized on day 13 by all treatments (Boyle and Shann, 1995). In the field, edaphic and plant factors contribute greatly to degradation rates. It is always important to screen the degradation rates of such compounds in association with the host so that the studies can be applied to field-scale mycoremediation.

12.10   PAH AND TNT DEGRADATION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and persistent environmental pollutants. They are highly toxic, mutagenic, and carcinogenic and constitute a great public health concern. The contamination produced by industries contains various classes of compounds and is highly complex. The

distribution of VAM propagules in India and reduction in diversity in coal, lignite, and calcite mine spoils compared to undisturbed soils have been studied (Ganesan et al., 1991). PAHs can disperse and be deposited on vegetation and soils from a range of industrial processes. VAM colonization was shown not to be influenced by anthracene addition in 10 g/kg soil in leek (Leyval and Binet, 1998). However, the percentage of colonization in clover and leek decreased upon addition of contaminated soil of 8 g/kg total PAHs from a coal production plant. Mycorrhizal fungi capable of degrading the diverse PAHs are limited and are noted in Table 12.4.

The ability to degrade PAHs (i.e., phenanthrene, chrysene, pyrene, and benzo[a] pyrene) has been tested by 16 species (27 strains) of ectomycorrhizal fungi (Braun-Lullemann et al., 1999). *Amanita muscaria*, *Paxillus involutus*, and *Suillis grevillei* utilized about 50% of phenanthrene. Strains of *Amanita excelsa*, *Leccinum versipelle*, *S. grevillei*, *S. luteus*, and *S. variegatus* removed about the same amount of benzo[a] pyrene within 4 weeks. *S. grevillei* removed nearly 50% pyrene, while *Boletus edulis* and *A. muscaria* removed 35% of chrysene. No correlation was shown to exist between the number of aromatic rings and the rate of metabolism as well as between the depletion of nitrogen and degradation. The rate of metabolism of benzo[a]pyrene is slow but very efficient, which correlates with the results obtained by white-rot fungi (Schutzendubel et al., 1999). The conversion rates of five PAHs in liquid culture by 58 fungi, including ECM fungi, have been noted (Gramss et al., 1999). Only one of 21 species does not degrade at least one PAH, and over half of the species tend to degrade all five PAHs. However, there is no information on the pathways of degradation of PAHs. All ECM fungal isolates are taken from unpolluted soil samples. It appears that the oxidative enzymes responsible for the degradation of these compounds may have some role in the ecology of ECM fungi. Active oxygen has been to be important for the oxidation of aromatic compounds by field-grown mats of ECM fungi (Gramss, 1997).

All ECM fungi (i.e., *Paxillus involutus*, two isolates of *Pisolithus tinctorius*, *Suillus variegatus*, and *Tylospora fibrillosa*) transform 2,4,6-trinitrotoluene (TNT) to a lesser or greater extent after 3 days of incubation in axenic cultures (Meharg et al., 1997b). *S. variegatus* produces the highest amount of biomass, and the rate of biotransformation is reduced under nitrogen-deficient conditions. TNT transformation capacity is higher in intact mycelium (600%) than in cultural filtrate containing extracellular enzymes. This may suggest the possibility of a number of pathways in TNT transformation by the intact mycelium. The dose–response curve for *S. variegatus* biomass production depicts fungal sensitivity to TNT. The TNT concentration reducing *S. variegatus* biomass by 50% (EC_{50}) is between 2 and 10 μg/ml, which is considered within the range (12 mg/g soil dry weight) for *Phanerochaete chrysosporium* (Spiker et al., 1992). This biotransformation occurs under conditions of sufficient carbon, which correlates very well with the requirement to have mycelium in symbiotic association with the plant.

TABLE 12.4 Polycyclic Aromatic Hydrocarbon-Degrading Capabilities of Mycorrhizal Fungi

Mycorrhizal Fungus	Biotest Type	Constituent	Degradation (%)	Duration (days/weeks)	Reference
ECM fungi					
Amanita muscaria	Complex liquid medium	Phenanthrene	50	4w	Braun-Lullemann et al., 1999
Paxillus involutus					
Suillus grevillei					
Boletus edulis		Chrysene	35		
Amanita muscaria					
Suillus grevillei		Pyrene	50		
Amanita excelsa		Benzo[*a*]pyrene	50		
Leccinum versipelle					
Suillus grevillei					
Suillus luteus					
Suillus variegatus					
Hebeloma crustuliniforme	Liquid culture	Phenanthrene	24	14d	Gramss et al., 1999
		Anthracene	29		
		Fluoranthene	52		
		Pyrene	51		
		Perylene	9		
Lactarius deliciosus		Phenanthrene	32		
		Anthracene	25		
		Fluoranthene	39		
		Pyrene	32		
		Perylene	3		

12.11 PCB DEGRADATION

Polychlorinated biphenyls (PCBs) are used widely in industry, due to insulating properties and resistance to burning. A large amount of PCB has been released into the environment due to improper industrial practices. ECM fungi are one of the biological agents that may be employed to solve this problem. The potential for survival of ectomycorrhizal fungi and colonization of plant roots at the contaminated sites has stimulated research on their application in PCB degradation. Fungi–plant symbiotic associations have a greater potential than bacteria and saprophytic fungi to survive at PCB-contaminated sites.

ECM fungi show degradation of 14 of 21 PCBs (Donnelly and Fletcher, 1995). At least 14 fungi metabolize 20% of PCBs. The number and relative amounts of PCB congener metabolized by fungi range from 0 to 7 of 19. Lower chlorinated congeners degrade more rapidly than higher chlorinated congeners. The average recovery is 78% based on the species and congener. *Gautieria crispa* and *Radiigera atrogleba* metabolize the highest number of congeners: five and seven, respectively. *Radiigera atrogleba* almost metabolizes two congeners and 60 to 79% of one congener, and *Hysterangium gardneri* degrades 80% of two congeners. PCBs are better degraded by *Suillus granulatus* than by *Hymenoscyphus ericae*.

Organofluorine compounds, some of which are agrochemicals, are highly toxic and accumulate in the environment, due to their persistent nature. *Tylospora fibrillosa* and other species degraded 4-fluorobiphenyl (4-FBP) in batch cultures in a study by Green et al. (1999b). Four major biotransformation products were detected by [14]HPLC profiles, and six major fluorine-containing products were recognized by ^{19}F-NMR spectroscopy. Two major products, 4-fluorobiphen-4′-ol and 4-fluorobiphen-3′-ol, were confirmed and there was no concrete evidence of identification of other products. Based on ^{19}F-NMR spectroscopy and [14]HPLC profiles, 4-FBP was degraded by *Thelephora terrestris* (>65% biotransformation products), *Suillus variegatus* (>50%), and *Hymenoscyphus ericae* (>20%), respectively. The transformation products formed were identical to those revealed by *T. fibrillosa*. More than 85% of 4-FBP was degraded during incubation with *T. fibrillosa* and other mycorrhizal fungi. However, 4-FBP was not biotransformed by *Suillus granulatus*. These results show the involvement of monoxygenation in the biotransformation. The occurrence of sequential hydroxylation in the mycorrhizal fungi was also determined. This was the first detailed and important study on the elucidation of knowledge on the mechanism of action describing intermediary products during degradation by mycorrhizal fungi.

12.12 HERBICIDE DEGRADATION

The potential of metabolizing herbicides by ECM fungi has been recognized to a certain extent. Several ECM fungi (i.e., *Hebeloma cylindrosporum*,

Suillus bellini, and *S. variegatus*) display the highest degradation of chlor-propham in the culture medium (Rouillon et al., 1989). About half of the cultures reveal the detection of 3-chloroaniline, an intermediate product after 1 week of incubation. Many ECM fungi have shown the ability to degrade two chlorinated herbicides, atrazine and 2,4-dichlorophenoxyacetic acid (2,4-D) (Donnelly et al., 1993; Donnelly and Fletcher, 1994). Among the mycor-rhizal fungi, *Rhizopogon vinicolor* revealed the maximum degradation of 1.0 mM 2,4-D alone when supplemented with 1 mM nitrogen concentration. The ERM fungus *H. ericae* showed the highest level of carbon into the tissue due to atrazine. In general, an increase in herbicide degradation correlated with an increase in nitrogen concentration. In most instances, degradation occurred by incorporation of herbicide carbon into tissue, not by mineraliza-tion. The degradation of side chains may have occurred but was not detected. [14]C-(ring)-labeled atrazine is not mineralized by soil fungi, but the fungal cells show 25% atrazine (Wolf and Martin, 1975). The biotransformation of 1-naphthalene acetic acid by *Pisolithus arhizus* (*tinctorius*) leads to the forma-tion of two major products, 1,2-dihydroxy-1,2-dihydronaphthalene-1-acetic acid γ-lactone and 4-hydroxy-1-naphthalene acetic acid (Soledade et al., 1994). A pesticide mecoprop, 2-(2-methyl-4-chlorophenoxy)propionic acid, is well degraded by a complex consortia of microbes in the rhizosphere (Lappin et al., 1985).

12.13   COMPARISON OF MYCORRHIZAL AND WHITE-ROT FUNGI

White-rot fungi produce extracellular oxidative lignin- and humic acid–degrading enzymes, which have contributed to the development of their use in mycoremediation. The best studied enzymes are LiP, MnP, and laccase. Extracellular release of lignin enzymes plays an important role in the myco-remediation of soils. These enzymes can diffuse to soil surfaces, where the persistent organic compounds tend to bind, thus increasing the bioavailability of these compounds. One significant advantage lies in the fact that contact between hyphae or mycelia of white-rot fungi and organic compounds is not necessarily required, which leads to degradation without toxicity. Extracel-lular enzymatic reactions reduce soil toxicity so that white-rot fungi can toler-ate high concentrations of these constituents. The tolerance of certain white-rot fungi to cyanide concentrations is 100 times greater than other soil organisms, leading to mineralization via ligninolytic enzymes (Barr and Aust, 1994).

Several researchers have tried to compare mycorrhizal and white-rot fungi. The degradation of aromatic compounds depends on the fungus, substrate, and physiological conditions. A number of mycorrhizal fungi have been shown to degrade aromatic xenobiotics in batch experiments, including the pesticides atrazine and 2,4-D and the lower chlorinated PCBs. In this way,

these are similar to white-rot fungi in the degradation of a wide range of aromatic xenobiotics, whereas mycorrhizal fungi depend on the host plant for carbon. Cairney and Burke (1994) reported the production of certain enzyme-degrading components of plant cell walls by mycorrhizal fungi. Some mycorrhizal fungi are known to induce ligninolytic enzymes in vitro (Griffiths and Caldwell, 1992). The degradative ability of PCBs by mycorrhizal fungi is equaled or exceeded by that of the white-rot fungus *Phanerochaete chrysosporium* (Donnelly and Fletcher, 1995). In some studies, mycorrhizal fungi and *Phanerochaete chrysosporium* revealed none or enhanced degradation of aromatic xenobiotics due to increased carbon and/or nitrogen addition (Donnelly et al., 1993; Yadav et al., 1995). Low nitrogen and carbon additions favor the production of ligninolytic (Griffiths and Caldwell, 1992) and plant cell wall–degrading fungal enzymes (Cairney and Burke, 1994). However, Meharg et al. (1997a) reported degradation of 2,4-dichlorophenol by *Suillus variegatus* and *Paxillus involutus* under increased carbon concentration in culture experiments and during symbiosis with the host plant. However, the mycorrhizal fungi show more rapid mineralization in symbiosis with the host plant than that of mycelium in batch experiments. This implies that ligninolytic enzymes are not a requirement for the initial steps in degradation by mycorrhizal fungi. A wide range of bacteria and fungi have been shown to degrade TNT. It is evident that the mechanism of initial steps is more or less similar throughout these organisms. This entails the formation of hydroxyl-aminodinitrotoluenes by reduction of one of the nitro groups on the aryl ring and the formation of aminodinitrotoluenes by further reduction (Higson, 1992; Gorontzy et al., 1994). It has been concluded that plasmalemma redox potential causes reduction of TNT by *Phanerochaete chrysosporium* (Stahl and Aust, 1993). Intact cells of *Suillus variegatus* showed more efficiency in biotransformation than the possible reduction of TNT by the cultural filtrate (Meharg et al., 1997b). The mycorrhizal and wood-rotting fungi produced extracellular enzymes that can be related to the degradation of certain constituents. The results also indicate ring fission and the ultimate production of carbon dioxide in the pathways of biotransformation for both mycorrhizal and white-rot fungi (Vyas et al., 1994; Yadav et al., 1995; Dietrich et al., 1995).

Mycorrhizal and wood-rotting fungi differ in ecological functions in diverse ecosystems. Wood-rotting fungi are wood-decomposing saprophytic fungi growing on dead organic matter, and mycorrhizal fungi are symbiotic associations of plant roots. The degradation of lignin and cellulose by white-rot fungi and to a certain extent by brown-rot fungi is now well established. Bending and Read (1997) reported on the low abilities to degrade lignin and phenolics by mycorrhizal fungi relative to wood-rotting fungi, together with the ecological significance of the results. Degradation of lignin and phenolics by mycorrhizal fungi has implications for its nutrition and that of the host plant. Several reports suggest the production of peroxidase enzyme activities by ECM and ERM fungi during axenic cultures, but no evidence has been shown; however,

peroxidase activities may be established under certain conditions in the future.

12.14   CONCLUSIONS AND FUTURE PERSPECTIVES

Mycorrhizas constitute a bridge for nutrient transport from soils to plant roots. Their role in the formation of soil aggregates and the protection of host plants against drought and root pathogens is well established. A slow process of remediation by these fungi is predicted, which depends on the development of the host root system and associated fungal biomass. The selection of compatible host–fungus–substrate combinations can be exploited by inoculating with ECM or ERM fungi for sustaining the infections. A survival rate of years is well known when inoculated experimentally with ECM fungi (Selosse et al., 1999). Multitrophic interactions are useful in rapid remediations. Fast remediation of persistent organic compounds may be possible with interaction between ECM fungi or bacteria.

High variability and lack of homogeneity in experimental protocols, bioassays, and field data are quite familiar in mycorrhizal research. Variability in concentration, duration, and exposure of constituent, coupled with soil and edaphic features, multiple constituents, short-lived/long-lived infections, and colonization and greenhouse/natural conditions will greatly affect the generation of data. Such inconsistencies make interpretations and conclusions more difficult. A fungus growing continuously in the field indicates an ample supply of carbon coming from the host plant. This can lead to the development of certain enzyme activities and a fungal biomass that aids in the degradation of toxic compounds. Experiments should be designed so as to decipher the intricate fungal degradations in the field in symbiotic association with the host plant as to how the influence of environmental factors on degradation and the degradation rate are affected.

Due to prevailing mycorrhizal fungal diversity in ecosystems, it is difficult to predict the effect of soil acidification, metal or hydrocarbon pollution, and other factors on mycorrhizal communities. Because of this effect, the loss of fungal species may occur in soils, resulting in increased predisposition of the host plant to environmental stress. Current knowledge on the functional diversity of mycorrhizal communities is fragmentary. Therefore, the ultimate interest shifts back to the effects of pollutants on the extramatrical mycelial systems of these fungi in soil. This can result in reduction or gain of fungal biomass in soil. Study of the effects of pollutants on the growth of extramatrical mycelium of these fungi is the first step in understanding the stability or sustainability in the ecosystems. This knowledge on the behavior of such fungi in association with the host plant can be deciphered collectively. Ultrastructural details related to soil acidification and contamination, nutrient transfer, and structural alterations can answer questions of general ecological importance. Trees can be planted on soils low in contamination where there is no risk of human health. Trees can be inoculated with the suitable fungi. This may

result in gradual decontamination after the establishment of mycorrhizal fungi in the soil. Important gaps exist in understanding multitrophic interactions, and such complexity is far greater than described.

Little work has been performed on screening of these fungi related to persistent organic compounds, and comprehensive screening is required. Mineralization of certain compounds has been demonstrated in certain cases, and the intermediate products are known in only a few cases. Production of extracellular enzymes by mycorrhizal fungi is now known and has a great potential in soil mycoremediation. Unfortunately, no example is known that elucidates the mechanism of action of mycotransformation of any pollutant. It is necessary to identify matching host plant species, and efforts need to be intensified to perform large-scale greenhouse field trials to update the process. Soil characteristics related to contaminants, fungal mycelia, host plants, and the efficiency of the process of mycoremediation need to be optimized. Tree growth and its management, including fertilization and irrigation, will also be important in the future of rhizosphere mycoremediation (Meharg and Cairney, 2000a).

VAM fungi can express anastomosis formation and nuclear and protoplasmic exchange. Genetic exchange during anastomosis formation can open the door to vegetative compatability in natural populations of VAM fungi. Somatic incompatibility (Dahlberg, 1999) and genetic transformation in ECM fungi (Lemke et al., 1999) have been described. Success can be obtained by cloning transport system genes in plants by complementation of yeast mutants (D'Enfert et al., 1995). This will assist in our understanding of the molecular aspects of transport systems in ectomycorrhizal fungi and ectomycorrhizas. Transport-system-specific antibody development against protein sequences derived from cDNAs or against purified transport proteins can be helpful in this regard (Chalot and Brun, 1998).

Genetic transformation system is highly complex, due to the symbiotic nature of these fungi. However, it is now possible to design molecular techniques to characterize signals, genes, and proteins necessary for symbiosis. Only then can the involvement of these molecules and signals in the ectomycorrhizal process be demonstrated functionally. Genetically engineered mycorrhizal fungi can be developed and applied for the manipulation of host plants in many physiological and ecological ways. Such novel fungi can express metal-chelating factors for the reduced uptake of soluble metals by roots. In addition, an adverse environment around root systems due to acidity or toxic pollutants can be changed.

REFERENCES

Agerer, A. (1999) Anatomical characteristics of identified ectomycorrhizas: an attempt towards a natural classification. In: *Mycorrhiza Structure, Function, Molecular Biology and Biotechnology*, A. Varma, and B. Hock, eds. Springer-Verlag, Berlin, pp. 633–682.

Aggangan, N.S, B. Dell, and N. Malajczuk (1998) Effects of chromium and nickel on growth of the ectomycorrhizal fungus *Pisolithus* and formation of ectomycorrhizas on *Eucalyptus urophylla* S.T. Blake. *Geoderma* **84**: 15–27.

Allen, M.F. (1991) *The Ecology of Mycorrhizae.* Cambridge University Press, Cambridge.

Allen, E.B., M.F. Allen, D.J. Helm, J.M. Trappe, R. Molina, and E. Rincon (1995) Patterns and regulation of mycorrhizal plant and fungal communities. *Plant Soil* **170**: 47–62.

An, Z.-Q., J.W. Hendix, D.E. Hershman, and G.T. Henson (1990) Evaluation of the most probable number (MPN) and wet-sieving methods for determining soil-borne populations of endogonaceous mycorrhizal fungi. *Mycologia* **82**: 576–581.

Aruguete, D.H., J.H. Aldstadt III, and G.M. Mueller (1998) Accumulation of several heavy metals and lanthanides in mushrooms (Agaricales) from the Chicago region. *Sci. Total Environ.* **224**: 43–56.

Barr, D.P., and S.D. Aust (1994) Mechanisms white rot fungi use to degrade pollutants. *Environ. Sci. Technol.* **28**: 79–87.

Bending, G.D., and D.J. Read (1995) The structure and function of the vegetative mycelium of ectomycorrhizal plants. VI. Activities of nutrient mobilizing enzymes in birch litter colonized by *Paxillus involutus* (Fr.) Fr. *New Phytol.* **130**: 411–417.

Bending, G.D., and D.J. Read (1996a) Effects of the soluble polyphenol tannic acid on the activities of ericoid and ectomycorrhizal fungi. *Soil Biol. Biochem.* **28**: 1595–1602.

Bending, G.D., and D.J. Read (1996b) Nitrogen mobilization from tannin–protein complexes by ericoid and ectomycorrhizal fungi. *Soil Biol. Biochem.* **28**: 1603–1612.

Bending, G.D., and D.J. Read (1997) Lignin and soluble phenol degradation by ectomycorrhizal and ericoid mycorrhizal fungi. *Mycol. Res.* **101**: 1348–1354.

Berthelsen, B.O., R.A. Olsen, and E. Steinnes (1995) Ectomycorrhizal heavy metal accumulation as a contributing factor to heavy metal levels in organic surface soils. *Sci. Total Environ.* **170**: 141–149.

Blaudez, D., B. Botton, and M. Chalot (2000) Cadmium uptake and subcellular compartmentation in the ectomycorrhizal fungus *Paxillus involutus*. *Microbiology* **146**: 1109–1117.

Bonello, P., T.D. Bruns, and M. Gardes (1998) Genetic structure of a natural population of the ectomycorrhizal fungus *Suillus pungens*. *New Phytol.* **138**: 533–542.

Bougher, N.L. (1994) Diversity of ectomycorrhizal fungi associated with eucalyptus in Australia. In: *Mycorrhizas for Plantation Forestry in Asia*, M. Brundrett, B. Dell, N. Malajczuk, and G. Mingqin, eds. Australian Centre for International Agriculture Research, Canberra, Australia, pp. 8–15.

Boyle, J.J., and J.R. Shann (1995) Biodegradation of phenol, 2,4-DCP, 2,4-D and 2,4,5-T in field-collected rhizosphere and nonrhizosphere soils. *J. Environ. Qual.* **24**: 782–785.

Braun-Lullemann, A., A. Huttermann, and A. Majcherczyk (1999) Screening of ectomycorrhizal fungi for degradation of polycyclic aromatic hydrocarbons. *Appl. Microbiol. Biotechnol.* **53**: 127–132.

Brooks, R.R. (1998) General introduction. In: *Plants That Hyperaccumulate Heavy Metals*, R.R. Brooks, ed. CAB International, Wallingford, UK, pp. 1–14.

Brunner, I., B. Frey, and T.K. Riesen (1996) Influence of ectomycorrhization and cesium/potassium ratio on uptake and translocation of cesium in Norway spruce seedlings. *Tree Physiol*. **16**: 705–711.

Bruns, T.D., and M. Gardes (1993) Molecular tools for the identification of ectomycorrhizal fungi: taxon-specific oligonucleotide probes for suilloid fungi. *Mol. Ecol*. **2**: 233–242.

Burgess, T., P. Laurent, B. Dell, N. Malajczuk, and F. Martin (1995) Effect of the fungal isolate aggressivity on the biosynthesis of symbiosis-related polypeptides in differentiating eucalypt ectomycorrhiza. *Planta* **195**: 408–417.

Burke, R.M., and J.W.G. Cairney (1998) Carbohydrate oxidases in ericoid and ectomycorrhizal fungi: a possible source of Fenton radicals during the degradation of lignocellulose. *New Phytol*. **139**: 637–645.

Burke, R.M., and J.W.G. Cairney (2002) Laccases and other polyphenol oxidases in ecto- and ericoid mycorrhizal fungi. *Mycorrhiza* **12**: 105–116.

Cabello, M.N. (1997) Hydrocarbon pollution: its effect on native arbuscular mycorrhizal fungi (AMF). *FEMS Microbiol. Ecol*. **22**: 233–236.

Cabello, M.N. (2001) Mycorrhizas and hydrocarbons. In: *Fungi in Bioremediation*, G.M. Gadd, ed. Cambridge University Press, Cambridge, pp. 456–471.

Cairney, J.W.G., and R.M. Burke (1994) Fungal enzymes degrading plant cell walls: their possible significance in the ectomycorrhizal symbiosis. *Mycol. Res*. **98**: 1345–1346.

Cairney, J.W.G., and R.M. Burke (1996) Plant cell wall-degrading enzymes in ericoid and ectomycorrhizal fungi. In: *Mycorrhizas in Integral Systems*, C. Azcon-Aguilar and J.M. Barea, eds. European Commission, Brussels, Belgium, pp. 218–221.

Cairney, J.W.G., and R.M. Burke (1998) Do ecto- and ericoid mycorrhizal fungi produce peroxidase activities? *Mycorrhiza* **8**: 61–65.

Cairney, J.W.G., and A.A. Meharg (1999) Influences of anthropogenic pollution on mycorrhizal fungal communities. *Environ. Pollut*. **106**: 169–182.

Chalot, M., and A. Brun (1998) Physiology of organic nitrogen acquisition by ectomycorrhizal fungi and ectomycorrhizas. *FEMS Microbiol. Rev*. **22**: 21–44.

Chambers, S.M., R.M. Burke, P.R. Brooks, and J.W.G. Cairney (1999) Molecular and biochemical evidence for manganese-dependent peroxidase activity in *Tylospora fibrillosa*. *Mycol. Res*. **103**: 1098–1102.

Chappelka, A.H., J.S. Kush, G.B. Runion, S. Meier, and W.D. Kelly (1991) Effects of soil-applied lead on seedling growth and ectomycorrhizal colonisation of Loblolly pine. *Environ. Pollut*. **72**: 307–316.

Chen, D.M., A.F.S. Taylor, R.M. Burke, and J.W.G. Cairney (2001) Identification of genes for lignin peroxidases and manganese peroxidases in ectomycorrhizal fungi. *New Phytol*. **152**: 151–158.

Clint, G.M., and J. Dighton (1992) Uptake and accumulation of radiocaesium by mycorrhizal and non-mycorrhizal heather plants. *New Phytol*. **121**: 555–561.

Colpaert, J.V., and J.A. Van Assche (1992) Zinc toxicity in ectomycorrhizal *Pinus sylvestris*. *Plant Soil* **143**: 201–211.

Colpaert, J.V., and J.A. Van Assche (1993) The effects of cadmium on ectomycorrhizal *Pinus sylvestris* L. *New Phytol.* **123**: 325–333.

Colpaert, J.V., and A. Van Laere (1996) A comparison of the extracellular enzyme activities of two ectomycorrhizal and a leaf-saprophytic basidiomycete colonizing beach leaf litter. *New Phytol.* **133**: 133–141.

Colpaert, J.V., and K.K. Van Tichelen (1996) Mycorrhizas and environmental stress. In: *Fungi and Environmental Change*, J. Frankland, ed. Cambridge University Press, Cambridge, pp. 109–128.

Colpaert, J.V., P. Vandenkoornhuyse, K. Adriaensen, and J. Vangronsveld (2000) Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillus luteus*. *New Phytol.* **147**: 367–379.

Dahlberg, A. (1999) Somatic incompatibility in ectomycorrhizas. In: *Mycorrhiza Structure, Function, Molecular Biology and Biotechnology*, A. Varma and B. Hock, eds. Springer-Verlag, Berlin, pp. 111–132.

De Araujo, A.A., and S. Roussos (2002) A technique for mycelial development of ectomycorrhizal fungi on agar media. *Appl. Biochem. Biotechnol.* **98–100**: 311–318.

D'Enfert, C., M. Minet, and F. Lacroute (1995) Cloning plant genes by complementation of yeast mutants. *Methods Cell Biol.* **49**: 417–430.

Denny, H.J., and I. Ridge (1995) Fungal slime and its role in the mycorrhizal amelioration of zinc toxicity to higher plants. *New Phytol.* **130**: 251–257.

Denny, H.J., and D.A. Wilkins (1987) Zinc tolerance in *Betula* spp. IV. The mechanism of ectomycorrhizal amelioration of zinc toxicity. *New Phytol.* **106**: 545–554.

Di Bonito, R., M.L. Elliott, and E.A. Des Jardin (1995) Detection of an arbuscular mycorrhizal fungus in roots of different plant species with the PCR. *Appl. Environ. Microbiol.* **61**: 2809–2810.

Dietrich, D., W.J. Hickey, and R. Lamar (1995) Degradation of 4,4′-dichlorophenyl, 3,3′, 4,4′-tetrachlorobiphenyl, and 2,2′, 4,4′, 5,5′-hexachlorobiphenyl by the white-rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **61**: 3904–3909.

Dighton, J., and A.E. Jansen (1991) Atmospheric pollutants and ectomycorrhizae: more questions than answers. *Environ. Pollut.* **73**: 179–204.

Dittmann, J., H. Wolfgang, and H. Bucking (2002) Biodegradation of aromatic compounds by white rot and ectomycorrhizal fungal species and the accumulation of chlorinated benzoic acid in ectomycorrhizal pine seedlings. *Chemosphere* **49**: 297–306.

Dixon, R.K., and C.A. Buschena (1988) Response of ectomycorrhizal *Pinus banksiana* and *Picea glauca* to heavy metals in soil. *Plant Soil* **105**: 265–271.

Donnelly, P.K., and J.S. Fletcher (1994) Potential use of mycorrhizal fungi as bioremediation agents. In: *Bioremediation Through Rhizosphere Technology*, T.A. Anderson and J.D. Coats, eds. American Chemical Society, Washington, DC, pp. 93–99.

Donnelly, P.K., and J.S. Fletcher (1995) PCB metabolism by ectomycorrhizal fungi. *Bull. Environ. Contam. Toxicol.* **54**: 507–513.

Donnelly, P.K., J.A. Entry, and D.L. Crawford (1993) Degradation of atrazine and 2,4-dichlorophenoxyacetic acid by mycorrhizal fungi at three nitrogen concentrations in vitro. *Appl. Environ. Microbiol.* **59**: 2642–2647.

Douds, D.D., Jr., and P.D. Millner (1999) Biodiversity of arbuscular mycorrhizal fungi in agroecosystems. *Agric. Ecosyst. Environ.* **74**: 77–93.

Douds, D.D., Jr., and G. Nagahashi (2000) Signaling and recognition events prior to colonization of roots by arbuscular mycorrhizal fungi. In: *Current Advances in Mycorrhizae Research*, G.K. Podila and D.D. Douds, Jr., eds. APS Press, St. Paul, MN, pp. 1–10.

Egerton-Warburton, L.M., and B.J. Griffin (1995) Differential responses of *Pisolithus tinctorius* isolates to aluminium *in vitro*. *Can. J. Bot.* **73**: 1229–1233.

Entry, J.A., P.K. Donnelly, and K. Cromack, Jr. (1992) The influence of carbon nutrition on *Armillaria ostoyae* growth and phenolic degradation. *Eur. J. For. Pathol.* **22**: 149–156.

Entry, J.A., P.T. Rygiewicz, and W.H. Emmingham (1994) ^{90}Sr uptake by *Pinus ponderosa* and *Pinus radiata* seedlings inoculated with ectomycorrhizal fungi. *Environ. Pollut.* **86**: 201–206.

Entry, J.A., L.S. Watrud, and M. Reeves (1999) Accumulation of ^{137}Cs and ^{90}Sr from contaminated soil by three grass species inoculated with mycorrhizal fungi. *Environ. Pollut.* **104**: 449–457.

Fawaris, B.H., and K.J. Johanson (1995) Fractionation of caesium (^{137}Cs) in coniferous forest soil in central Sweden. *Sci. Total Environ.* **170**: 221–228.

Fomina, M.A., I.J. Alexander, J.V. Colpaert, and G.M. Gadd (2005) Solubilization of toxic metal minerals and metal tolerance of mycorrhizal fungi. *Soil Biol. Biochem.* **37**: 851–866.

Frey, B., A. Vilarino, M. Schuepp, and J. Arines (1994) Chitin and ergosterol content of extraradical and intraradical mycelium of the vesicular–arbuscular mycorrhizal fungus *Glomus intraradices*. *Soil Biol. Biochem.* **26**: 711–717.

Galli, U., M. Meier, and C. Brunold (1993) Effects of cadmium on nonmycorrhizal and mycorrhizal Norway spruce seedlings and its ectomycorrhizal fungus *Laccaria laccata* Bk and Br: sulfate reduction, thiols and distribution of the heavy metal. *New Phytol.* **125**: 837–843.

Ganesan, V., S. Ragupathy, B. Parthipan, D.B. Rajini Rani, and A. Mahadevan (1991) Distribution of vesicular-arbuscular mycorrhizal fungi in coal, lignite and calcite mine spoils of India. *Biol. Fertil. Soils* **12**: 131–136.

Gaspar, M.L., M.N. Cabello, M.C. Cazau, and R.J. Pollero (2002) Effect of phenanthrene and *Rhodotorula glutinis* on arbuscular mycorrhizal fungus colonization of maize roots. *Mycorrhiza* **12**: 55–59.

Gehring, C.A., T.C. Theimer, T.G. Whitham, and P. Kein (1998) Ectomycorrhizal fungal community structure of pinyon pine communities growing in two environmental extremes. *Ecology* **79**: 1562–1572.

Giltrap, N.J. (1982) Production of polyphenol oxidases by ectomycorrhizal fungi with special reference to *Lactarius* spp. *Trans. Br. Mycol. Soc.* **78**: 75–81.

Giovannetti, M., and B. Mosse (1980) An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytol.* **84**: 489–499.

Gonzalez-Chavez, C. (2000) Arbuscular mycorrhizal fungi from As/Cu polluted soils, contribution to plant tolerance and importance of external mycelium. Ph.D. dissertation, University of Reading, UK.

Gonzalez-Chavez, M.C., R. Carrillo-Gonzalez, S.F. Wright, and K.A. Nichols (2004) The role of glomalin, a protein produced by arbuscular mycorrhizal fungi, in sequestering potentially toxic elements. *Environ. Pollut.* **130**: 317–323.

Goodman, D.M., D.M. Durall, J.A. Trofymow, and S.M. Berch, eds. (1996) *A Manual of Concise Descriptions of North American Ectomycorrhizae*. Mycologue Publications and Canadian Forest Service, Victoria, AB, Canada.

Gorontzy, T., O. Drzyzga, M.W. Kahl, J. Bruns-Nagel, et al. (1994) Microbial degradation of explosives and related compounds. *Crit. Rev. Microbiol.* **20**: 265–284.

Graham, J.H., N.C. Hodge, and J.B. Morton (1995) Fatty acid methyl ester profiles for characterization of Glomalean fungi and their endomycorrhizae. *Appl. Environ. Microbiol.* **61**: 58–64.

Gramss, G. (1997) Activity of oxidative enzymes in fungal mycelia from grasslands and forest soils. *J. Basic Microbiol.* **6**: 407–423.

Gramss, G., T. Gunther, and W. Fritsche (1998) Spot tests for oxidative enzymes in ectomycorrhizal, wood- and litter decaying fungi. *Mycol. Res.* **102**: 67–72.

Gramss, G., B. Kirsche, K.-D. Voigt, Th. Gunther, and W. Fritsche (1999) Conversion rates of five polycyclic aromatic hydrocarbons in liquid cultures in fifty-eight fungi and the concomitant production of oxidative enzymes. *Mycol. Res.* **103**: 1009–1018.

Gray, S.N., J. Dighton, S. Olsson, and D.H. Jennings (1995) Real-time measurement of uptake and translocation of ^{137}Cs within mycelium of *Schizophyllum commune* Gr. by autoradiography followed by quantitative image analysis. *New Phytol.* **129**: 449–465.

Green, H., J. Larsen, P.A. Olsson, D.F. Jensen, and I. Jakobsen (1999a) Suppression of the biocontrol agent *Trichoderma harzianum* by mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* in root-free soil. *Appl. Environ. Microbiol.* **65**: 1428–1434.

Green, N.A., A.A. Meharg, C. Till, J. Troke, and J.K. Nicholson (1999b) Degradation of 4-fluorobiphenyl by mycorrhizal fungi as determined by ^{19}F nuclear magnetic resonance specroscopy and ^{14}C radiolabeling analysis. *Appl. Environ. Microbiol.* **65**: 4021–4027.

Grellier, B., D.G. Strullu, F. Martin, and S. Renaudin (1989) Synthesis in-vitro micro-analysis and phosphorus-31 NMR study of metachromatic granules in birch mycorrhizas. *New Phytol.* **112**: 49–54.

Griffiths, R.P., and B.A. Caldwell (1992) Mycorrhizal mat communities in forest soils. In: *Mycorrhizas in Ecosystems*, D.J. Read, D.H. Lewis, A.H. Fitter, and I.J. Alexander, eds. CAB International, Wallingford, UK, pp. 98–105.

Gruhn, C.M., and J.R. Miller (1991) Effect of Cu on tyrosinase activity and poly-amine content of some ectomycorrhizal fungi. *Mycol. Res.* **95**: 268–272.

Guidot, A., J.-C. Debaud, and R. Marmeisse (2002) Spatial distribution of the below-ground mycelia of an ectomycorrhizal fungus inferred from specific quantification of its DNA in soil samples. *FEMS Microbiol. Lett.* **42**: 477–486.

Gunther, Th., B. Perner, and G. Gramss (1998) Activities of phenol oxidising enzymes of ectomycorrhizal fungi in axenic culture and in symbiosis with Scots pine (*Pine sylvestris* L). *J. Basic Microbiol.* **38**: 197–206.

Hahn, A., C. Gobel, and B. Hock (1999) Immunochemical properties of mycorrhizas. In *Mycorrhiza Structure, Function, Molecular Biology and Biotechnology*, A. Varma and B. Hock, eds. Springer-Verlag, Berlin, pp. 177–204.

Harley, J.L., and S.E. Smith (1983) *Mycorrhizal Symbiosis*. Academic Press, London.

Hartley, J. (1997) Effects of heavy metal pollution on Scots pine (*Pinus sylvestris* L.) and its ectomycorrhizal symbionts. Ph.D. dissertation, University of Leeds, Leeds, UK.

Hartley, J., J.W.G. Cairney, and A.A. Meharg (1997) Do ectomycorrhizal fungi exhibit adaptive tolerance to potentially toxic metals in the rhizosphere? *Plant Soil* **189**: 303–319.

Haselwandter, K., and G.D. Bowen (1996) Mycorrhizal relations in trees for agroforestry and land rehabilitation. *For. Ecol. Manag.* **81**: 1–17.

Haselwandter, K., M. Berreck, and P. Brunner (1988) Fungi as bioindicators of radio-caesium contamination: pre- and post-Chernobyl activities. *Trans. Br. Mycol. Soc.* **90**: 171–174.

Haselwandter, K., O. Bobleter, and D.J. Read (1990) Degradation of ^{14}C-labelled lignin and dehydropolymer of coniferyl alcohol by ericoid and ectomycorrhizal fungi. *Arch. Microbiol.* **153**: 352–354.

Haselwandter, K., C. Leyval, and F.E. Sanders (1994) Impact of arbuscular mycorrhizal fungi on plant uptake of heavy metals and radionuclides from soil. In: *Impact of Arbuscular Mycorrhizas on Sustainable Agriculture and Natural Ecosystems*, S. Gianinazzi and H. Schuepp, eds. Birkhauser, Basel, Switzerland, pp. 179–189.

Heggo, A., J.S. Angle, and R.L. Chaney (1990) Effects of vesicular–arbuscular mycorrhizal fungi on heavy metals uptake by soybeans. *Soil Biol. Biochem.* **22**: 865–869.

Hetrick, B.A.D., G.W.T. Wilson, and D.A.H. Figge (1994) The influence of mycorrhizal symbiosis and fertilizer amendments on establishment of vegetation in heavy-metal mine spoil. *Environ. Pollut.* **86**: 171–179.

Higson, F.J. (1992) Microbial degradation of nitroaromatic compounds. *Adv. Appl. Microbiol.* **37**: 1–19.

Howe, R., R.L. Evans, and S.W. Ketteridge (1997) Copper-binding proteins in ectomycorrhizal fungi. *New Phytol.* **135**: 123–131.

Hutchinson, L.J. (1990) Studies on the systematics of ectomycorrhizal fungi in axenic culture. III. Patterns of polyphenol oxidase activity. *Mycologia* **82**: 424–435.

Ianson, D.C., and M.F. Allen (1986) The effects of soil texture on extraction of vesicular–arbuscular mycorrhizal spores from arid soils. *Mycologia* **78**: 164–168.

Ingleby, K., P.A. Mason, F.T. Last, and L.V. Fleming (1990) *Identification of Ectomycorrhizas*. Institute of Terrestrial Ecology Research Publication 5. HMSO, London.

Jones, M.D., D.M. Durall, and P.B. Tinker (1991) Fluxes of carbon and phosphorus between symbionts in willow ectomycorrhizas and their changes with time. *New Phytol.* **119**: 99–106.

Jonsson, L., A. Dahlberg, M.-C. Nilsson, O. Zachrisson, et al. (1999a) Ectomycorrhizal fungal communities in late-successional boreal forests and composition following wildfire. *Mol. Ecol.* **8**: 205–215.

Jonsson, T., S. Kokaji, R. Finlay, and S. Erland (1999b) Ectomycorrhizal community structure in a limited spruce forest. *Mycol. Res.* **103**: 501–508.

Kanunfre, C.C., and G.T. Zancan (1998) Physiology of exolaccase production by *Thelephora terrestris. FEMS Microbiol. Lett.* **161**: 151–156.

Khalil, S., L. Carpenter-Boggs, and T.E. Loynachan (1994) Procedure for rapid recovery of vam fungal spores from soil. *Soil Biol. Biochem.* **26**: 1587–1588.

Kieliszewska-Rokicka, B., M. Rudawska, and T. Leski (1997) Ectomycorrhizae of young and mature Scots pine trees in industrial regions of Poland. *Environ. Pollut.* **98**: 315–324.

Kim, S.-J., J. Zheng, S.T. Hiremath, and G.K. Podila (1998) Cloning and characterization of a symbiosis-related gene from an ectomycorrhizal fungus *Laccaria bicolor. Gene* **222**: 203–212.

Kirchner, G. (1998) Applicability of compartmental models for simulating the transport of radionuclides in soil. *J. Environ. Radioact.* **38**: 339–352.

Kong, F.X., Y. Liu, W. Hu, P.P. Shen, C.L. Zhou, and L.S. Wang (2000) Biochemical responses of the mycorrhizae in *Pinus massoniana* to combined effects of Al, Ca and low pH. *Chemosphere* **40**: 311–318.

Kottke, I., X.M. Qian, K. Pritsch, I. Haug, and F. Oberwinkler (1998) *Xerocomus badius–Picea abies*, an ectomycorrhiza of high activity and element storage capacity in acidic soil. *Mycorrhiza* **7**: 267–275.

Kreuzinger, N., R. Podeu, F. Gruber, F. Gobl, and C.P. Kubicek (1996) Identification of some ectomycorrhizal basidiomycetes by PCR amplification of their gpd (glyceraldehyde-3-phosphate dehydrogenase) genes. *Appl. Environ. Microbiol.* **62**: 3432–3438.

Krishna, K.R. (2004) *Mycorrhizas: A Molecular Analysis.* Science Publishers, Enfield, NH.

Kuek, C. (1994) Issues concerning the production and use of inocula of ectomycorrhizal fungi in increasing the economic productivity of plantations. In: *Management of Mycorrhizas in Agriculture, Horticulture and Forestry*, A.D. Robson, L.K. Abbott, and N. Malajczuk, eds. Kluwer, Dordrecht, The Netherlands, pp. 221–230.

Lacourt, I., S. D'Angelo, M. Girlanda, K. Turnau, P. Bonfante, et al. (2000) Genetic polymorphism and metal sensitivity of *Oidiodendron maius* strains isolated from polluted soils. *Ann. Microbiol.* **50**: 157–166.

Landeweert, R., E. Hoffland, R.D. Finlay, T.W. Kuyper, and N.V. Breemen (2001) Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. *Trends Ecol. Evolut.* **16**: 248–254.

Landeweert, R., C. Veenman, T.W. Kuyper, H. Fritze, K. Wernars, and E. Smit (2003) Quantification of ectomycorrhizal mycelium in soil by real-time PCR compared to conventional quantification techniques. *FEMS Microbiol. Ecol.* **45**: 283–292.

Lanfranco, L., A. Bolchi, E.C. Ros, S. Ottonello, and P. Bonfante (2002) Differential expression of a metallothionein gene during the presymbiotic versus the symbiotic phase of an arbuscular mycorrhizal fungus. *Plant Physiol.* **130**: 58–67.

Lappin, H.M., M.P. Greaves, and J.H. Slater (1985) Degradation of the herbicide mecoprop (2-(2-methyl-4-chlorophenoxy)propionic acid) by a synergistic microbial community. *Appl. Environ. Microbiol.* **49**: 429–433.

Leake, J.R. (1987) Metabolism of phyto- and fungitoxic phenolic acids by the ericoid mycorrhizal fungus. In: *Mycorrhizae in the Next Decade: Practical Applications and Research Priorities—Proceedings of the Seventh North American Conference on Mycorrhizae*, May 3–8, Gainesville, FL, D.M. Sylvia, L.L. Hung, and J.H. Graham, eds. Publis, Gainesville, FL, 332 pp.

Leake, J.R., and D.J. Read (1991) Proteinase activity in mycorrhizal fungi. III. Effects of protein, protein hydrolysate, glucose and ammonium on production of extracellular proteinase in *Hymenoscyphus ericae* Read Korf and Kernan. *New Phytol.* **116**: 309–318.

Lemke, P.A., N.K. Singh, and U.A. Temann (1999) Genetic transformation in ectomycorrhizal fungi. In: *Mycorrhiza Structure, Function, Molecular Biology and Biotechnology*, A. Varma and B. Hock, eds. Springer-Verlag, Berlin, pp. 133–152.

Leyval, C., and P. Binet (1998) Effect of polyaromatic hydrocarbons in soil on arbuscular mycorrhizal plants. *J. Environ. Qual.* **27**: 402–407.

Li, X., and P. Christie (2001) Changes in soil solution Zn and pH and uptake of Zn by arbuscular mycorrhizal red clover in Zn-contaminated soil. *Chemosphere* **42**: 201–207.

Liao, J.P., X.G. Lin, Z.H. Cao, Y.Q. Shi, and M.H. Wong (2003) Interactions between arbuscular mycorrhizae and heavy metals under sand culture experiment. *Chemosphere* **50**: 847–853.

Linkov, I., and W.R. Schell (1999) *Contaminated Forests: Recent Developments in Risk Identification and Future Perspectives.* Kluwer, Amsterdam, The Netherlands.

Ma, W.K., S.D. Siciliano, and J.J. Germida (2005) A PCR-DGGE method for detecting arbuscular mycorrhizal fungi in cultivated soils. *Soil Biol. Biochem.* **37**: 1589–1597.

Madan, R., C. Pankhurst, B. Hawke, and S. Smith (2002) Use of fatty acids for identification of AM fungi and estimation of the biomass of AM spores in soil. *Soil Biol. Biochem.* **34**: 125–128.

Maijala, P., K.V. Fagerstedt, and M. Raudaskoski (1991) Detection of extracellular cellulolytic and proteolytic activity in ectomycorrhizal fungi and *Heterobasidion annosum* (Fr.) Bref. *New Phytol.* **117**: 643–648.

Markkola, A.M., R. Ohthonen, O. Tarvainen, and U. Ahonen-Jonnarth (1995) Estimates of fungal biomass in Scots pine stands on an urban pollution gradient. *New Phytol.* **131**: 139–147.

Marks, G.C., and T.T. Kozlowski (1973) *Ectomycorrhizae: Their Ecology and Physiology.* Academic Press, London.

Marschner, P., D.L. Godbold, and G. Jentschke (1996) Dynamics of lead accumulation in mycorrhizal and non-mycorrhizal Norway spruce (*Picea abies* (L.) Karst). *Plant Soil* **178**: 239–245.

Martino, E., S. Perotto, R. Parsons, and G.M. Gadd (2003) Solubilization of insoluble inorganic zinc compounds by ericoid mycorrhizal fungi derived from heavy metal polluted sites. *Soil Biol. Biochem.* **35**: 133–141.

McGonigle, T.P., M.H. Miller, D.G. Evan, G.L. Fairchild, and J.A. Swan (1990) A new method which gives an objective measure of colonization of roots by vesicular–arbuscular fungi. *New Phytol.* **115**: 495–501.

Meharg, A.A. (1994) Integrated tolerance mechanisms: constitutive and adaptive plant responses to elevated metal concentrations in the environment. *Plant Cell Environ.* **17**: 989–993.

Meharg, A.A., and J.W.G. Cairney (2000a) Ectomycorrhizas: extending the capabilities of rhizosphere remediation? *Soil Biol. Biochem.* **32**: 1475–1484.

Meharg, A.A., and J.W.G. Cairney (2000b) Co-evolution of mycorrhizal symbionts and their hosts to metal contaminated environments. *Adv. Ecol. Res.* **30**: 69–112.

Meharg, A.A., J.W.G. Cairney, and N. Maguire (1997a) Mineralization of 2,4-dichlorophenol by ectomycorrhizal fungi in axenic culture and in symbiosis with pine. *Chemosphere* **34**: 2495–2504.

Meharg, A.A., G.R. Dennis, and J.W.G. Cairney (1997b) Biotransformation of 2,4,6-trinitrotoluene (TNT) by ectomycorrhizal basidiomycete. *Chemosphere* **35**: 513–521.

Miller, O.K., A.E. Linkins, and M.A. Chmielewski (1978) Fungal biomass responses in oil perturbed tundra at Barrow Alaska. *Arctic* **31**: 394–407.

Molina, R., and J.G. Palmer (1982) Isolation, maintenance and pure culture manipulation of ectomycorrhizal fungi. In: *Methods and Principles of Mycorrhizal Research*, N.C. Schenck, ed. APS Press, St. Paul, MN, pp. 115–129.

Molina, R., H. Massicotte, and J.M. Trappe (1992) Specificity phenomena in mycorrhizal symbiosis: community-ecological consequences and practical implications. In: *Mycorrhizal Functioning*, M.F. Allen, ed. Chapman & Hall, London, pp. 357–423.

Moore, A.E.P., H.B. Massicotte, and R.L. Peterson (1989) Ectomycorrhiza formation between *Eucalyptus pilularis* Sm. and *Hydnangium carneum* Wallr. in Dietr. *New Phytol.* **112**: 193–204.

Morselt, A.F.W., W.T.M. Smits, and T. Limonard (1986) Histochemical demonstration of heavy metal tolerance in ectomycorrhizal fungi. *Plant Soil* **96**: 417–420.

Morton, J.B., M. Franke, and S.P. Bentivenga (1999) Development foundations for morphological diversity among endomycorrhizal fungi in Glomales. In: *Mycorrhiza Structure, Function, Molecular Biology and Biotechnology*, A. Varma and H. Hock, eds. Springer-Verlag, Berlin, pp. 617–632.

Mukerji, K.G., C. Manoharachary, and B.P. Chamola, eds. (2002) *Techniques in Mycorrhizal Studies.* Kluwer, Amsterdam, The Netherlands.

Nicolotti, G., and S. Egli (1998) Soil contamination by crude oil: impact on the mycorrhizosphere and on revegetation potential of forest tress. *Environ. Pollut.* **99**: 37–43.

Nikolova, I., K.J. Johanson, and A. Dahlberg (1997) Radiocaesium in fruitbodies and mycorrhizae in ectomycorrhizal fungi. *J. Environ. Radioact.* **37**: 115–125.

Nikolova, I., K.J. Johanson, and S. Clegg (2000) The accumulation of ^{137}Cs in the biological compartment of forest soils. *J. Environ. Radioact.* **47**: 319–326.

Norris, J.R., D.J. Read, and A.K. Varma (1991) *Methods in Microbiology*, Vol. 23. Academic Press, London.

Norris, J.R., D.J. Read, and A.K. Varma (1992) *Methods in Microbiology*, Vol. 24. Academic Press, London.

Olsson, P.A., E. Baath, I. Jakobsen, and B. Soderstrom (1996) Soil bacteria respond to presence of roots but not to mycelium of arbuscular mycorrhizal fungi. *Soil Biol. Biochem.* **28**: 463–470.

Olsson, P.A., I. Thingstrup, I. Jakobsen, and E. Baath (1999) Estimation of the biomass of arbuscular mycorrhizal fungi in a linseed field. *Soil Biol. Biochem.* **31**: 1879–1887.

Pierleoni, R., M. Buffalini, L. Vallorani, et al. (2004) *Tuber borchii* fruit body: 2-dimensional profile and protein identification. *Phytochemistry* **65**: 813–820.

Podila, G.K., and D.D. Douds, Jr., eds. (2000) *Current Advances in Mycorrhizae Research*. APS Press, St. Paul, MN.

Rafferty, B., D. Dawson, and A. Kliashtorin (1997) Decomposition in two pine forests: the mobilisation of ^{137}Cs and K from forest litter. *Soil Biol. Biochem.* **29**: 1673–1681.

Rafferty, B., M. Brennan, D. Dawson, and D. Dowding (2000) Mechanisms of ^{137}Cs migration in coniferous forest soils. *J. Environ. Radioact.* **48**: 131–143.

Rajapakse, S., and J. Miller (1991) Methods for studying vesicular–arbuscular mycorrhizal root colonization and related root physical properties. *Methods Microbiol.* **24**: 301–316.

Ramarao, P. (2002*) Fundamentals of Mycorrhizal Biology and Biotechnology*. Agrotech Publishing Company, Udaipur, Rajasthan, India.

Read, D.J. (1999) Mycorrhiza: the state of the art. In: *Mycorrhiza Structure, Function, Molecular Biology and Biotechnology*, A. Varma and B. Hock, eds. Springer-Verlag, Berlin, pp. 3–36.

Read, D.J., and R. Boyd (1986) Water relations of mycorrhizal fungi and their host plants. In: *Water, Fungi and Plants*, P. Ayers and L. Boddy, eds. Cambridge University Press, Cambridge, pp. 287–303.

Rodriguez-Tovar, A.V., R. Ruiz-Medrano, A. Herrera-Martinez, et al. (2005) Stable genetic transformation of the ectomycorrizal fungus *Pisolithus tinctorius*. *J. Microbiol. Methods* **63**: 45–54.

Rogers, R.D., and S.E. Williams (1986) Vesicular–arbuscular mycorrhizae: influence on plant uptake of cesium and cobalt. *Soil Biol. Biochem.* **4**: 371–376.

Rouillon, R., C. Poulain, J. Bastide, and C.M. Coste (1989) Degradation of the herbicide chlorpropham by some ectomycorrhizal fungi in pure culture. *Agric. Ecosyst. Environ.* **28**: 421–424.

Rousseau, J.V.D., D.M. Sylvia, and A.J. Fox (1994) Contribution of ectomycorrhiza to the potential nutrient-absorbing surface of pine. *New Phytol.* **128**: 639–644.

Ruhm, W., L. Kammerer, L. Hiersche, and E. Wirth (1996) Migration of ^{137}Cs and [134]Cs in different forest soil layers. *J. Environ. Radioact.* **33**: 63–75. Erratum (1997) *J. Environ. Radioact.* **34**: 103–106.

Ruhm, W., S. Yoshida, Y. Muramatsu, M. Steiner, and E. Wirth (1999) Distribution patterns for stable [133]Cs and their implication with respect to the long-term fate of radioactive [134]Cs and ^{137}Cs in a semi-natural ecosystem. *J. Environ. Radioact.* **45**: 253–270.

Salzer, P., and T. Boller (2000) Elicitor induced reactions in mycorrhizae and their suppression. In: *Current Advances in Mycorrhizae Research*, G.K. Podila and D.D. Douds, Jr., eds. APS Press, St. Paul, MN, pp. 1–10.

Salzer, P., B. Hubner, A. Sirrenberg, and A. Hager (1997) Differential effect of purified spruce chitinases and β-1,3 glucanases on the activity of elicitors from ectomycorrhizal fungi. *Plant Physiol.* **114**: 957–968.

Sarand, I., S. Timonen, E.-L. Nurmiaho-Lassila, T. Koivila, K. Haahtela, et al. (1998) Microbial biofilms and catabolic plasmid harbouring degradative fluorescent pseudomonads in Scots pine mycorrhizospheres developed on petroleum contaminated soil. *FEMS Microbiol. Ecol.* **27**: 115–126.

Sarand, I., S. Timonen, T. Koivila, R. Peltola, and K. Haahtela, et al. (1999) Tolerance and biodegradation of *m*-toluate by Scots pine, a mycorrhizal fungus and fluorescent pseudomonads individually and under associative conditions. *J. Appl. Microbiol.* **86**: 817–826.

Sawyer, N.A., S.M. Chambers, and J.W.G. Cairney (1999) Molecular investigation of genetic distribution and genetic variation of *Cortinarius rotundisporus* in eastern Australian sclerophyll forests. *New Phytol.* **141**: 561–568.

Scheidegger, C., and I. Brunner (1999) Electron microscopy of ectomycorrhiza: methods, applications, and findings. In: *Mycorrhiza Structure, Function, Molecular Biology and Biotechnology*, A. Varma and B. Hock, eds. Springer-Verlag, Berlin, pp. 205–224.

Schell, W.R., I. Linkov, C. Myttenaere, and B. Morel (1996) A dynamic model for evaluating radionuclide distribution in forests from nuclear accidents. *Health Phys.* **70**: 318–335.

Schenck, N.C., and Y. Perez (1990) *Manual for the Identification of VA Mycorrhizal Fungi*, 3rd ed. Synergistic Publications, Gainesville, FL.

Schmitz, O., G. Danneberg, B. Hundeshagen, A. Klinger, and H. Bothe (1991) Quantification of vesicular–arbuscular mycorrhiza by biochemical parameters. *J. Plant Physiol.* **139**: 106–114.

Schutzendubel, A., A. Majcherczyk, C. Johannes, and A. Huttermann (1999) Degradation of fluorene, anthracene, phenanthrene, fluoranthene, and pyrene lacks connection to the production of extracellular enzymes by *Pleurotus ostreatus* and *Bjerkandera adusta. Int. Biodeterior. Biodegrad.* **43**: 93–100.

Selosse, M.-A., F. Martin, D. Bouchard, and F. le Tacon (1999) Structure and dynamics of experimentally introduced and naturally occurring *Laccaria* sp. discrete genotypes in a Douglas fir plantation. *Appl. Environ. Microbiol.* **65**: 2006–2016.

Sharples, J.M., A.A. Meharg, S.M. Chambers, and J.W.G. Cairney (2001) Arsenate resistance in the ericoid mycorrhizal fungus *Hymenoscyphus ericae. New Phytol.* **151**: 265–270.

Simon, L., R.C. Levesque, and M. Lalonde (1992) Rapid quantitation of PCR of endomycorrhizal fungi colonizing roots. *PCR Methods Appl.* **2**: 76–80.

Smith, S.E., and D.J. Read (1997) *Mycorrhizal Symbiosis*, 2nd ed. Academic Press, London.

Smith, G.W., and H.D. Skipper (1979) Comparison of methods to extract spores of vesicular–arbuscular mycorrhizal fungi. *Soil Sci. Soc. Am. J.* **43**: 722–725.

Soledade, M., C. Pedras, B.J. McAfee, and M.S. Lapp (1994) Biotransformation of 1-naphthalene acetic acid by the ectomycorrhizal fungus *Pisolithus arhizus*. *Phytochemistry* **36**: 825–827.

Spiker, J.K., D.L. Crawford, and R.L. Crawford (1992) Influence of 2,4,6-trinitrotoluene (TNT) concentration on the degradation of TNT in explosive-contaminated soils by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **58**: 3199–3202.

Stahl, J.D., and S.D. Aust (1993) Plasma membrane dependent reduction of 2,4,6-trinitrotoluene by *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Commun.* **192**: 471–476.

Steiner, M., I. Linkov, and S. Yoshida (2002) The role of fungi in the transfer and cycling of radionuclides in forest ecosystems. *J. Environ. Radioact.* **58**: 217–241.

Sutton, J.C., and G.L. Barron (1972) Population dynamics of *Endogone* spores in soil. *Can. J. Bot.* **50**: 1909–1914.

Timonen, S., and R. Sen (1998) Heterogeneity of fungal and plant enzyme expression in intact Scots pine–*Suillus bovinus* and –*Paxillus involutus* mycorrhizospheres developed in natural forest humus. *New Phytol.* **138**: 355–366.

Tommerup, I.C. (1982) Airstream fractionation of vesicular–arbuscular mycorrhizal fungi: concentration and enumeration of propagules. *Appl. Environ. Microbiol.* **44**: 533–539.

Tomsett, A.B. (1993) Genetics and molecular biology of metal tolerance in fungi. In: *Stress Tolerance of Fungi*, D.H. Jennings, ed. Marcel Dekker, New York, pp. 69–128.

Trappe, J.M. (1996) What is a mycorrhiza? In: *Proceedings of the Fourth European Symposium on Mycorrhiza*, EC Report EUR 16728, Granada, Spain, pp. 3–9.

Trojanowski, J., K. Haider, and A. Huttermann (1984) Decomposition of ^{14}C-labelled lignin, holocellulose and lignicellulose by mycorrhizal fungi. *Arch. Microbiol.* **139**: 202–206.

Turnau, K., I. Kottke, and F. Oberwinkler (1993) *Paxillus involutus–Pinus sylvestris* mycorrhizae from heavily polluted forest. I. Element localization using electron energy loss spectroscopy and imaging. *Bot. Acta* **106**: 213–219.

Turnau, K., I. Kottke, and J. Dexheimer (1996) Toxic element filtering in *Rhizopogon roseolus*/*Pinus sylvestris* mycorrhizas collected from calamine dumps. *Mycol. Res.* **100**: 16–22.

van Breemen, N., et al. (2000) Mycorrhizal weathering: a true case of mineral plant nutrition? *Biogeochemistry* **49**: 53–67.

Varma, A. (1998) *Mycorrhiza Manual.* Springer-Verlag, Berlin.

Varma, A. (1999) Hydrolytic enzymes from arbuscular mycorrhizae: the current status. In: *Mycorrhiza Structure, Function, Molecular Biology and Biotechnology*, A. Varma and B. Hock, eds. Springer-Verlag, Berlin, pp. 373–390.

Varma, A., and B. Hock, eds. (1999) *Mycorrhiza Structure, Function, Molecular Biology and Biotechnology.* Springer-Verlag, Berlin.

Vilarino, A., and J. Arines (1990) An instrumental modification of Gerdemann and Nicholson's method for extracting VAM fungal spores from soil samples. *Plant Soil* **121**: 211–216.

Vinichuk, M.M., K.J. Johanson, and A.F.S. Taylor (2004) ^{137}Cs in the fungal compartment of Swedish forest soils. *Sci. Total Environ.* **323**: 243–251.

Vyas, B.R.M., V. Sasek, M. Mutacha, and M. Bubner (1994) Degradation of 3,3′,4,4′-tetrachlorobiphenyl by selected white rot fungi. *Chemosphere* **28**: 1127–1134.

Wiemken, V., and T. Boller (2002) Ectomycorrhiza: gene expression, metabolism and wood-wide web. *Curr. Opin. Plant Biol.* **5**: 355–361.

Wolf, D.C., and J.P. Martin (1975) Microbial decomposition of ring-^{14}C atrazine, cyanuric acid, and 2-chloro-4,6-diamino-*s*-triazine. *J. Environ. Qual.* **4**: 134–139.

Wright, S.F., J.B. Morton, and J.E. Sworobuk (1987) Identification of a vesicular–arbuscular mycorrhizal fungus by using monoclonal antibodies in an enzyme-linked immunosorbent assay. *Appl. Environ. Microbiol.* **53**: 2222–2225.

Yadav, J., J.F. Quensen, J.M. Tiedje, and C.A. Reddy (1995) Degradation of polychlorinated biphenyl mixtures (Aroclors 1242, 1254, and 1260) by the white rot fungus *Phanerochaete chrysosporium* as evidenced by congener-specific analysis. *Appl. Environ. Microbiol.* **61**: 2560–2565.

Yokoyama, K., T. Tateishi, T. Marumoto, and M. Saito (2002) A molecular marker diagnostic of a specific isolate of an arbuscular mycorrhizal fungus, *Gigaspora margarita*. *FEMS Microbiol. Lett.* **212**: 171–175.

Yoshida, S., and Y. Muramatsu (1994) Accumulation of radiocaesium in basidiomycetes collected from Japanese forests. *Sci. Total Environ.* **157**: 197–205.

Yoshida, S., Y. Muramatsu, M. Steiner, M. Beli, A. Pasquale, B. Rafferty, et al. (2000) Relationship between radiocaesium and stable caesium in plants and mushrooms collected from forest ecosystems with different contamination levels. In: *Proceedings of the 10th International Congress of the International Radiation Protection Association,* May 2000, Hiroshima, Japan (P-11–244).

Zarb, J., and D.R. Walters (1995) Polyamine biosynthesis in the ectomycorrhizal fungus *Paxillus involutus* exposed to zinc. *Lett. Appl. Microbiol.* **21**: 93–95.

Zhu, Y., P. Christie, and A.S. Laidlaw (2001) Uptake of Zn by arbuscular mycorrhizal white clover from Zn-contaminated soil. *Chemosphere* **42**: 193–199.

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