Part I Shiitake

Chapter 2

Shiitake Spawn and Strain

PRESERVATION OF SHIITAKE SPAWN STOCKS BY CRYOGENIC STORAGE

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Introduction

The long-term preservation of the special properties and characteristics of a cultivated mushroom strain requires continual maintenance. The common method of continuous subculture[1] of strains on artificial media (the traditional method) provides an effective short-term preservation of mushroom strains. Yet this system can increase the risk of accidental contamination and/or changes in the morphological and physiological characteristics of the organisms (Jong and Davis, 1986). Continuous subculture is time consuming, laborious and impractical for large collections of mushroom strains.

Continuous Subculture: Strict hygiene and environmental control (dark storage at 4℃) should be maintained to avoid mycelial degeneration caused by factors such as pathogens, mutations and metabolic activities.

Pick the small edge of a colony and put it at the center of a petri dish or in the center or lower part of a test tube full of fresh media. When picking the sample, avoid the old media (the first inoculated, central part of the colony) that may now contain metabolic compounds that retard new mycelial growth. Browned parts should not be used. These new young healthy samples from the culture should be transferred to fresh media periodically. PDA (Potato Dextrose Agar) and YMA (Yeast Malt extract agar) are the usual media.

When the colony covers 70% of the media in the petri dishes, store them at 4-5℃ in darkness. They can be stored for 3-4 months in this manner before the next subculture is made.

Tubes (⌀ 18mm ×h150-180mm) should have a slanted media which is less than 30 degrees. When closed by a silicon cap, these tubes can be stored for 5-12 months at 8-15℃ and 60% relative humidity. Tubes can be stored for 3-6 months when sealed with a cotton cap.

Different long-term preservation methods have been developed in order to improve and preserve the genetic stability of strains. Today liquid nitrogen storage is accepted as the best preservation technique for long term storage of mushroom mycelia (Chvostová *et al*., 1995). This freezing process must be carefully managed in order for the mycelia to be frozen and successfully recovered. To avoid ice crystal formation and the cell damage caused by freezing in liquid nitrogen (-196 ℃), cryoprotective[2] solutions (cryoprotectants) are used.

The pioneer studies in this arena of fungal conservation at ultra-low temperatures were made before 1960 (Hwang, 1960). Since then, many different methods and materials have been evaluated in an effort to optimize the process. The viability of the frozen samples depends upon the mushroom species and the strain. Additionally, the age of mycelium, its growth conditions, the type of cryoprotectant being used and its rate penetration into the mushroom cells, and the methods and rate of freezing and thawing all affect the eventual success or failure (Chvostová *et al*., 1995; Mata *et al*., 2000).

The cryopreservation of superior mushroom strains is generally undertaken by cutting agar blocks from growing cultures

[1] Subculture: an act of producing another culture of microorganisms derived from an original culture

[2] Cryoprotective: serving to protect against the deleterious effects of subjection to freezing temperatures

and then immersing them in a cryoprotectant. After immersion in the cryoprotective solution, the agar blocks are gradually cooled from ambient temperature to -40 ℃ at a rate of 1-10 ℃/min (Smith, 1993, 1998) and then placed in liquid nitrogen. Both the gradual freezing of samples and the use of cryoprotective solutions have been considered absolutely necessary for the adequate recovery of the mycelia (Roquebert and Bury, 1993; Chvostová *et al*., 1995). However, good recovery of mycelia has been obtained with procedures using different spawn stocks, and different techniques.

For example *Agaricus bisporus* (the button mushroom) will grow mycelia from frozen spawn prepared with gramineous[3] seeds, when a pre-freezing procedure is used (Hwang and San Antonio, 1972, 1982; Kneebone *et al*., 1974; Jodon *et al*., 1982; Suman and Jandaik, 1991). Frozen mycelia have also been recovered for *Volvariella volvacea*, *Pleurotus* spp. and *Lentinula* spp. when the spawns were instantly frozen in cryoprotective solutions (Pérez and Salmones, 1997; Lara Herrera *et al*., 1998 a, b; Mata *et al*., 2000). It is very interesting to note that in the majority of the last cited cases, mycelial recovery and new growth were initiated from seed hila[4] or from fissures on the surface of the gramineous seeds. These results suggest that the seeds might have acted as mycelial protectors. In particular, although cellular contents are known to crystallize with rapid freezing, neither immediate freezing of the spawn nor the absence of cryoprotective substances appears to have been lethal in these studies. In support of this hypothesis, mycelia were recently successfully recovered from spawn rapidly frozen in liquid nitrogen without the use of cryoprotectant (Mata and Pérez Merlo, 2003). Although the exact mechanism by which the mycelium is protected by the spawn seeds remains unclear, the sample viability obtained with this method is very high (72-100%).

This paper shows the results of a developed methodology. It is a simple and inexpensive freezing system for conserving and recovering edible mushroom strains.

Spawn Preparation and the Freezing and Thawing of Samples

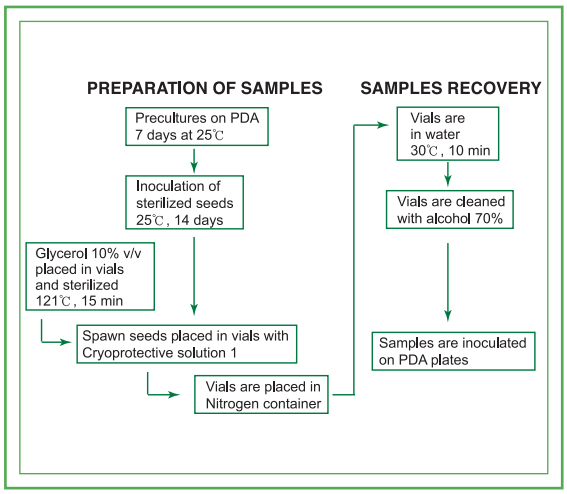

Figure 1. Important aspects of the method for spawn preservation by cryogenic storage

Shiitake strains must be pre-cultured for 7 days on petri dishes with a solid media of potato dextrose and agar (PDA). Figure 1 shows the most important aspects of the method for spawn preservation by cryogenic storage.

Spawn must be prepared by the conventional method with pre-treated sorghum seeds (*Sorghum vulgare*) hydrated to 65% and then sterilized at 121 ℃ for 1 hour. After sterilization the sorghum seeds must be placed in sterile petri dishes. Each dish must then be inoculated with a pre-cultured mycelium, plus its agar disc (±0.5cm in diameter). Inoculated dishes, sealed with elastic plastic film, must be incubated in darkness for 14 days (at 25 ℃), allowing mycelial growth to completely cover the sorghum grains.

Special polycarbonate vials (NALGENE): Polycarbonate boxes (for vial arranging inside the liquid nitrogen container) and gloves must be used for freezing in liquid nitrogen (Fig. 2). The cryoprotective solution is prepared with glycerol (10% v/v) and distilled water. The cryoprotective solution is placed into the vials and sterilized at 121 ℃ for 15 minutes. After cooling, the spawn of fully-incubated sorghum seeds must be placed in the vials (25 seeds per vial) (Figs. 3). The samples must remain in contact with the cryoprotective solution for 1 hour, and then the vials in their polycarbonate containers can be placed directly into the liquid nitrogen (Fig. 4).

For strain recuperation, the vials must be removed from the liquid nitrogen and thawed by submergence in distilled water at 30 ℃ for 10 min (Mata *et al*., 2000) (Fig. 5). Once thawed, the vials are cleaned for 1 min with an alcohol solution (70% v/v). After cleaning, the cryoprotectant is drained off and the seeds are placed in petri dishes with PDA in order to encourage mycelial recovery and growth. It is very important to carry out this part of the process in a sterile environment using a laminar flow hood.

[3] Gramineous: of, relating to, or characteristic of grasses
[4] Hila: sg. hilum. scars on a seed

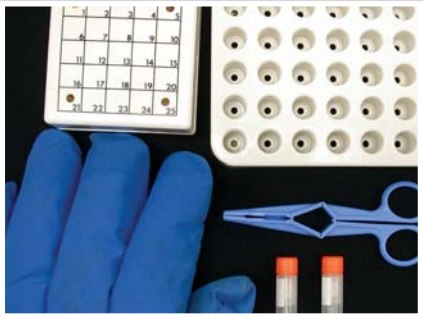

Figure 2. Material and tools used for freezing spawn stocks

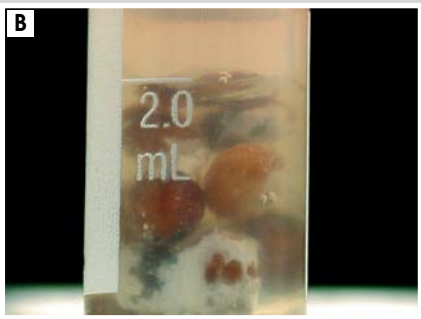

Figure 3. Spawn fully-incubated sorghum seeds in dishes (**A**) and placed in the vials with cryoprotectant (**B**)

Figure 4. Vials placed in polycarbonate containers directly frozen in liquid nitrogen

Mycelial Growth and Viability Tests

When this system is used, usually after thawing, all sorghum grains had lost the external mycelial layers that had covered them. The mycelia from all samples will usually be recovered from seed hila, or from fissures on the seed surfaces (Mata and Pérez-Merlo, 2003) (Figs. 6). The rate of recovery in seed samples varies according to the treatment and the species. Strains of *Pleurotus djamor*, *P. pulmonarius*, *P. columbinus*, *V. volvacea*, *Lentinula edodes* and *L. boryana* have showed 100% recovery (Mata and Pérez-Merlo, 2003; Mata *et al*., 2004). Factors such as the age and physiological state of the hyphae, as well as its cytoplasmic contents, may have affected the capacity of mushroom cells to resist freezing and thawing (Smith and Thomas, 1998). Previous studies (Lara Herrera *et al*., 1998a; Mata *et al*., 2000) showed that treating spawn stocks with a cryoprotective solution of glycerol, without pre-freezing, resulted in 100% recovery of *Pleurotus* and *Lentinula* strains. Furthermore, no differences in mushroom production were observed. Spawn viability of *Pleurotus* spp. strains was not affected by liquid nitrogen for 8 years. No significant differences were observed in mycelial growth rate or in mushroom morphology or size (Mata *et al*., 2004).

Figure 5. Thawing vials for strain recuperation

Figure 6. Mycelium recovered from seed hilium (**A**) and samples completely recovered after cryopreservation (**B**)

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