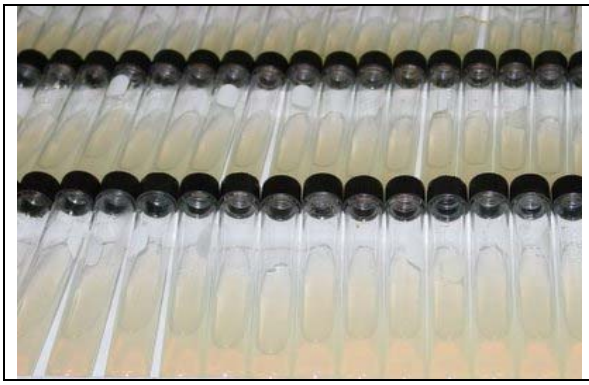
Care and handling of cultures of the cultivated mushroom (*Agaricus bisporus*)

Daniel J. Royse[1] and Vija Wilkinson[2]
[1]Professor and [2]Research Technologist, Department of Plant Pathology, The Pennsylvania State University, University Park, PA 16802



This paper provides background information on the care, maintenance and preparation of agar and cryogenic cultures of the cultivated mushroom, *Agaricus bisporus*. Methods for the initial phases of spawn production are given to help in the development of a reliable product. Care and attention in the early aspects of culture management will reduce the chances of later problems in the spawn production process.

Procuring cultures

Isolates of the cultivated mushroom used to make spawn are obtained by one of five basic means: hybridization, monospore, multispore, mycelial transfer, or tissue culture. Pure cultures of mushroom strains may also be obtained from a culture bank. These cultures usually are shipped as agar cultures in test tube slants or Petri dishes. Once the master culture is obtained, the proper maintenance and handling of this culture is of primary importance.

Mailing containers used to ship agar cultures should be opened immediately upon receipt to allow maximum air circulation and gas exchange. Cultures should not be stored in closed containers since volatile substances produced by the fungus can inhibit mycelial growth. Mushroom cultures distributed to growers are of uniform and normal type with growth rates similar to other isolates of the same strain. It is advisable to make note of the appearance of the tubes on arrival, particularly if the strain is to be sub-cultured.

Culture substrates

The most commonly used agar medium for maintaining pure cultures of the cultivated mushroom is potato dextrose yeast extract agar. Formulas for preparing this medium are given in Table 1.

Table 1 Potato dextrose yeast extract agar medium for maintenance of cultures of the cultivated mushroom.

1. Wash 250 g potatoes (do not peel).
2. Slice 3 mm thick.
3. Wash with tap water until the water is clear.
4. Cover with distilled water and cook until tender.
5. Drain liquid through flannel cloth or several thicknesses of cheesecloth into a flask.
6. Rinse potatoes once or twice with a little distilled water.
7. Keep liquids and throw potatoes away; add enough distilled water to make up on liter of liquid.
8. Bring liquid to a boil and add:
    a. 15 g agar – stir until dissolved (watch carefully or it will boil over).
    b. 10 g dextrose
    c. 1.5 g of yeast extract
9. Autoclave for 15 minutes at 121°C.

*Agar is a gelatinous substance, a white-to tan-colored powder obtained from certain seaweeds (red algae) and used as a solidifying agent; agar may be obtained from various commercial laboratories.

Agar cultures should be grown for 2 to 4 weeks to determine if their typical characteristics will remain stable before being used. In general, transfers should be made to fresh agar medium as soon as possible, usually within 1 to 2 weeks. The transfers may be taken from several areas in the tube or Petri dish, although the original plug of inoculum in the center of the tube should not be used for new transfers. The size of the inoculum plug should not exceed 3 x 3 mm to minimize the transfer of accumulated staling products in the old agar. Transfers from sectored areas should be avoided (see below).

The new cultures should be incubated at about 23°C for maximum growth. Temperatures between 0 and 23°C will not harm the culture but will slow the growth rate. The air temperature should not exceed 27°C, since higher temperatures slow the growth rate and may result in heat damage to the mycelium. Freezing of the mushroom mycelium on agar medium without cryoprotectants may damage or kill the mycelium and must be avoided.

It is advisable to observe the new transfers weekly for uniformity of growth, sectoring, contamination, or abnormal appearance. Four to 6-week old agar cultures may be stored at about 4°C for 4 months. Non-refrigerated agar cultures should be transferred at least every 60 days. Drying out may be minimized by using 14 ml of agar medium instead of 10 ml per standard test tube (20 x 150 mm).

It is important that mushroom cultures have free air circulation around the tube. Cultures should never be incubated or stored in a closed system, such as a refrigerator, without a fan to circulate fresh air. Storage time is limited because agar cultures dry out even when refrigerated. Growth becomes limited and sectoring or abnormal growth may occur as

the nutrients in the agar become exhausted. Finally, mushroom cultures should never be sealed in any way that prevents free air exchange through the plug or cap.

Sectoring and abnormalities in cultures

Sectoring is any type of mycelial growth that differs in appearance, growth rate and color from the typical appearance of a given strain. Sectoring is often seen as a rapidly growing area near the leading edge of growth in an agar slant, and often the sector will exhibit a different growth habit than the rest of the culture. A sector may or may not revert to normal growth. Other abnormalities that might appear in a culture are fluffy, aerial mycelium or a color change such as browning or darkening of the mycelium.

The causes of sectoring are not well understood. It is believed that sectoring represents some type of genetic change, perhaps loss of heterozygosity at specific loci, deheterokaryotization, somatic recombination, or chromosomal loss in the culture. At the present time, no method is available to determine the ultimate productivity of a culture on agar.

Cultures are propagated solely on the basis of cultural characteristics exhibited in an agar slant. These characteristics vary considerably from strain to strain, but experience has shown that transfers from typical, uniform cultures of a given strain will produce reliable spawn when handled properly. On the other hand, a sector or change in vegetative growth could affect the productivity of the culture to an unknown extent. Therefore, it is very important to recognize and avoid propagation of sectored mycelium.

Cryogenic Storage

The successful preservation of mushroom cultures is dependent upon capturing the full productivity of the original culture. Spontaneous and enzymatic degradation of the culture's cellular contents must be suspended while maintaining the molecular and macroscopic aspects of the culture. Cryogenic freezing of mushroom cultures provides such a method.

The suspending of biological and chemical activity of cellular contents occurs at temperatures of -130°C or below. These temperatures can be achieved and maintained by both liquid nitrogen and mechanical refrigeration. Both systems have advantages and disadvantages, but we have successfully used liquid nitrogen storage of mushroom cultures since the early 1980s, so the following descriptions are based on liquid nitrogen storage.

Cryoprotectants

A cryoprotectant is a substance that is used to protect biological tissue from damage during freezing. Common cryoprotectants include glycerol and dimethyl sulfoxide (DMSO). Both of these materials are penetrating cryoprotectants at physiological temperatures, meaning that they are able to move across cell membranes into the

cytoplasm. Cryoprotectants serve to help maintain osmotic equilibrium during the freezing process and decrease the damage caused by intracellular ice nucleation. Glycerol is commonly used to protect mushroom cultures from freeze damage. We commonly add a 1 ml sterile glycerol solution (10%) to 2 ml capacity cryotubes and then submerge a few (up to 6) mycelial plugs or spawn grains into the solution (Fig. 1A, 1B).

Controlled rate freezing

Control of the rate of freezing is critical to minimize cell injury of mushroom cultures. A freezing rate that is too fast results in intracellular ice nucleation that can be lethal to the cultures. On the other hand, a very slow freezing rate can result in cell death due the 'pickling' effects of long periods of exposure to hypertonic solutions. An optimal rate of freezing minimizes the exposure time to hypertonic conditions while minimizing the formation of intracellular ice.

The optimum rate of freezing for mushroom cultures is about -1°C/minute. This can be achieved by using a Nalgene® *Cryo Freezing Container* with isopropyl alcohol to provide the critical, repeatable cooling rate. The vials are placed into a holder and the holder is fitted into the container of isopropyl alcohol. The lid is then closed and the *Cryo Freezing Container* is placed into a -70°C mechanical freezer (Fig. 1C). The *Cryo Freezing Container* can hold up to eight vials and can withstand repeated freeze/thaw cycles.

After 4 hours at -70°C, the cultures have reached the target temperature. They are then moved to the liquid nitrogen storage boxes (Fig. 1D, 1E). These boxes are then placed into the vapor phase (area above the liquid nitrogen in the bottom of the refrigerator) of the liquid nitrogen refrigerator for permanent storage or until needed (Fig. 1F).

Recovery and thawing of cryovials may be accomplished by floating the vial in a 37°C water bath for about 2 minutes. After thawing, the contents may be transferred to an agar medium contained in Petri dishes for resumed growth of the culture. In order to drain the cryoprotectant from the mycelial plug or spawn grain, the dish may be incubated at an angle (slant).

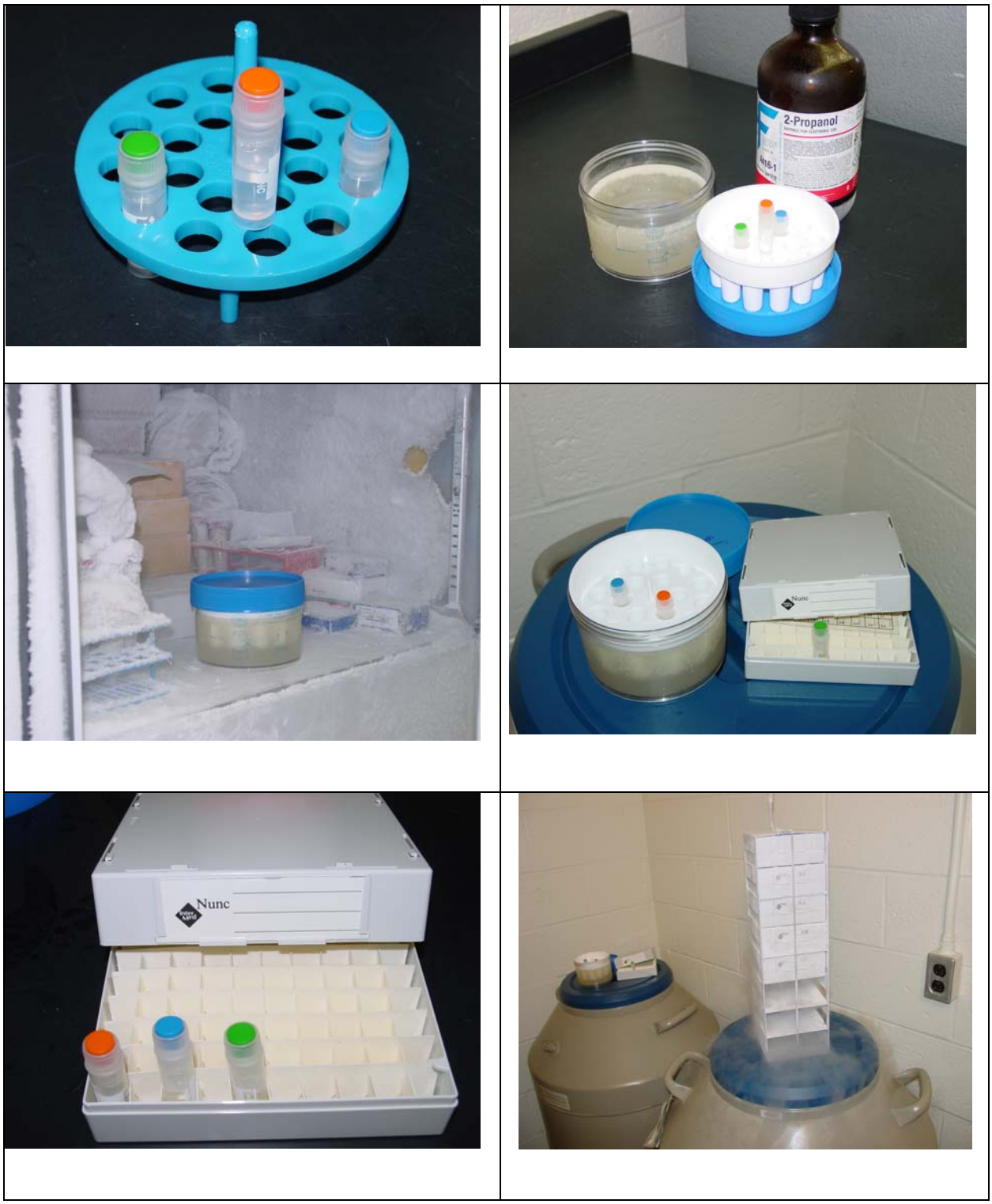


Figure 1, A-F. A) cryogenic vials containing 1 ml sterile 10% glycerol ready for receiving mycelial plugs, B) cryogenic vials with mycelial plugs placed into cryoholder, c) capped *Cryo Freezing Container* placed on shelf in -70°C mechanical freezer, d) cryovials removed from *Cryo Freezing Container* after 4 hr at -70°C and placed into cryoboxes (e) for final placement in liquid nitrogen refrigerator (f).

Liquid nitrogen freezers

Quality and static holding times for liquid nitrogen refrigerators have improved substantially over the last few years, as improved insulation and vacuum technology have been incorporated into their design. Static holding time for some refrigerator models are now as long as 340 days without requiring liquid nitrogen refill. Of course, the number of times the refrigerator is opened and samples are deposited and retrieved can affect the holding time. Refrigerator capacities vary considerably, so selecting a model to fit your applications may require some projection of long-term needs of the unit. Finally, the local availability of liquid nitrogen may influence the decision to purchase and use such refrigerators as a means to maintain cultures.

Inoculum-spawn production and storage

Grain selection – White millet (*Panicum miliaceum* L.) and rye (*Secale cereale* L.) grain are generally used in the U.S. for spawn production. Grain from the current crop year should be used to optimize freshness and viability of the kernels. Screening the grain to obtain uniform kernel size and to remove dust, cracked and immature kernels is essential to ensure a uniform product. Grain densities of 40 to 50 kernels/g and 180 to 200 kernels/g can be expected from rye and millet grain, respectively. Millet grain spawn has become more popular than rye grain spawn among mushroom growers in recent years because of the higher number of kernels/g. A higher kernel density results in a higher number of spawn inoculum points in compost.

Grain preparation – The preparation of millet and rye grain for spawn production may vary depending on the type of spawn manufacturing system used. Pre-cooking, or boiling in water for 20-30 min, of millet is generally required because the grain tends to become soft and sticky if water is added immediately prior to sterilization. Pre-cooking, followed by coating the millet with 2-3% (dry wt) precipitated calcium carbonate (PCC) before sterilization, minimizes the stickiness of the grain and provides a more flowable product. Rye grain, on the other hand, has a much thicker seed coat so precooking is not required and water and PCC can be added immediately before sterilization. Commercial spawn manufactures use V-blenders or horizontal blenders to mix and sterilize either the millet or rye grain in one operation, so the need for precooking is eliminated.

Incubation – The time needed to produce spawn varies and depends largely on the amount of inoculum, growing conditions, and the extent of mycelial growth desired. The flasks should be incubated until the grains are well colonized with mycelium, 12 to 18 days at 23°C. One 250 ml flask will inoculate five to six 1000 ml flasks, that also require between 12 to 18 days for desired mycelial growth. Shaking of flasks should be done every 4 to 7 days, depending on the growth variables indicated above. Frequent shaking increases the distribution of the mycelium throughout the grain, prevents compaction, and discourages sectoring.

Spawn storage – Grain should be well covered, but not overgrown, with mycelium before storage. Grain not full colonized with mycelium is easily invaded by contaminating

organisms. Spawn may be refrigerated at about 4°C for up to 6 months, although 4 months or less is preferable. Mycelium continues to grow at a reduced rate in cold storage. Approximately one day's growth at 23°C is equivalent to about one week's growth at 4°C. Therefore, prolonged storage of spawn may lead to compaction, sectoring, or other problems.

Summary

The importance of proper care and handling of mushroom cultures cannot be overemphasized. The initial phases of spawn production, including transfer of mycelium from agar to agar, agar to grain flask, and grain flask to final spawn container, are most critical. Abnormal or undesirable changes due to mishandling of cultures during any of these phases are likely to be perpetuated in the spawn, resulting in an unreliable product. Careful attention to the details of culture management in the initial stage should result in a more uniform, reliable spawn.

Further reading

American Type Culture Collection. 1991. Preservation methods: freezing and freeze-drying, 2nd ed. American Type Culture Collection, Rockville, Md.

Harvey, C., S. Lodder, R. Moore, and C. Kelly. 1998. A multidiscipline approach to spawn consistency. Mushroom News 46(7):6-9.

Reviewed by:

Gary Moorman, professor of plant pathology, The Pennsylvania State University
Donald D. Davis, professor of plant pathology, The Pennsylvania State University