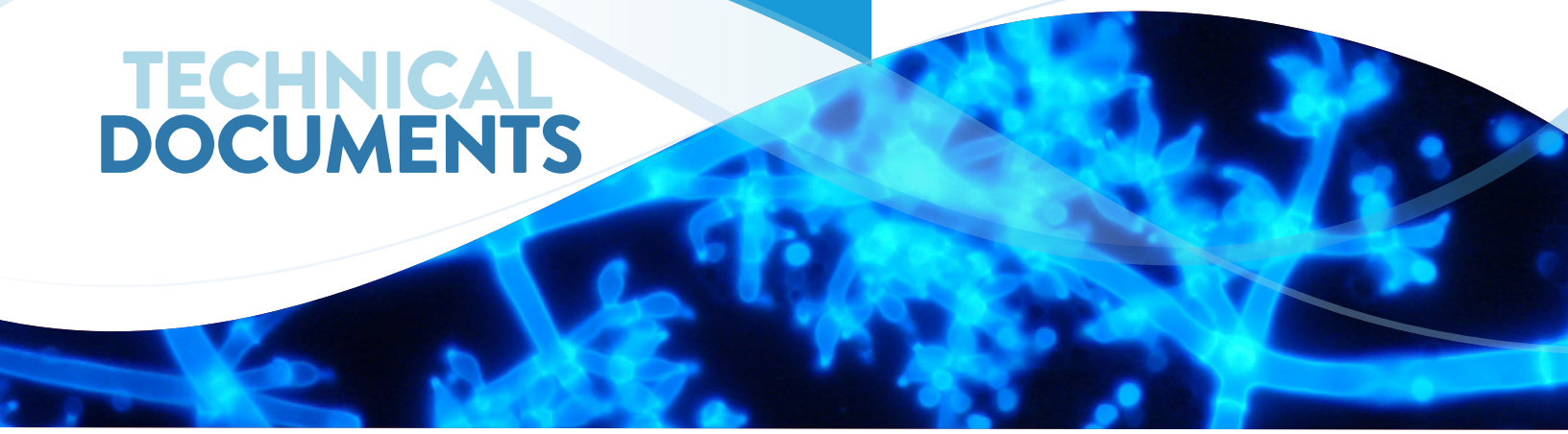

PRESERVATION AND RECOVERY OF FILAMENTOUS FUNGI

INTRODUCTION

Preservation methods for filamentous fungi vary depending on the type and degree of sporulation. Spore-forming strains (with the exception of zoosporic fungi) can usually be freeze-dried successfully. Similar success with nonsporulating strains is far less likely. Both types can be frozen and stored for long periods in liquid nitrogen or liquid nitrogen vapor. All plasmid-containing or mutant strains should be frozen directly from the original material if possible to prevent any alteration or loss of desired characteristics.

The following overview discusses preservation methods used at ATCC and is presented for descriptive purposes only; it is not intended as a laboratory protocol. Anyone planning to preserve cultures by these methods is strongly advised to study detailed, published protocols before proceeding.

FREEZE-DRYING SPOREFORMERS

PREPARING THE CULTURE

1. Grow fungi under conditions that will induce maximum sporulation so that sufficient spores will survive the freezing and drying process. Optimum media and growth conditions are listed in the strain descriptions in ATCC's online catalog at www.atcc.org. Literature cited there may also give further guidance on appropriate cultivation procedures.
2. Prepare a 20% solution of skim milk and autoclave at 116°C for 20 minutes in 10 mL tubes. One tube is usually more than enough for 10 freeze-dried vials unless the culture is very mycelial. Store the milk solution at 2-8°C until needed so that it will be cold when used.
3. Prepare the spore suspension by slowly introducing about 2 mL of milk solution into the culture tube or plate while gently scraping the surface of the culture with a pipette. Take care to avoid raising a cloud of spores, especially with *Aspergillus*, *Penicillium*, and other fungi that produce large amounts of dry spores. For example, *Neurospora* spores are very difficult to contain. It is therefore recommended that cultures for freeze-drying are grown on agar in 250 mL Erlenmeyer flasks.
4. Transfer the suspension back into the tube containing the remainder of the skim milk solution and mix thoroughly. If more than one plate or tube is used, repeat the procedure for each and pool the suspensions into one tube. A concentration of at least 10^6 spores per mL of milk solution is needed.
5. Dispense 0.2 mL of the suspension into each vial for freeze-drying. Many spores will begin to germinate when suspended in liquid, so timing is critical when filling vials. Spores should not be in the skim milk solution for more than two hours before being processed. Refrigerate filled vials while waiting for further processing.

FREEZE-DRYING METHODS

Although the process is somewhat labor-intensive, freeze-drying spore-forming fungi greatly facilitates their distribution and storage. The following four methods are described in detail in Simione and Brown (1991).

1. **Component freeze-dryer**—Samples are freeze-dried in cotton plugged glass inner vials which are then sealed inside glass outer ampules under vacuum. The components of the system (vacuum pump and condenser) are assembled on a benchtop.
2. **Commercial freeze-dryer**—Samples are prepared in glass vials in ampules (as in no. 1 above), and then freeze-dried in a commercial freeze-dryer.
3. **Serum vial**—Samples are processed in glass serum vials sealed with rubbers stoppers and metal caps. A commercial freeze-dryer is used.
4. **Manifold**—Samples are processed in bulbshaped or tubular glass ampules attached with latex tubing to a manifold. (This method is not used at ATCC, but is relatively inexpensive and uses equipment that a lab may already own.)

RECOVERY

1. If the culture to be recovered was obtained directly from ATCC, check it thoroughly upon receipt. If it is found to be unsatisfactory in any respect, notify ATCC so that the strain in question can be investigated.
2. Open the ampule as directed and, using sterile distilled water and a sterile pipette, transfer the contents of the preparation to approximately 5 mL of sterile distilled water in a test tube.
3. Allow the contents to rehydrate for at least one hour (two hours is better, and overnight is not too long) before transfer of a few drops to broth or agar. Use the media and growth conditions specified in the strain descriptions when first subculturing to ensure optimal recovery.
4. Incubate at the appropriate temperature. (The remainder of the suspension may be stored for a few days if refrigerated, allowing for another recovery attempt if the first should fail.) Given proper treatment and conditions, most cultures will grow in a few days. However, some may exhibit a prolonged lag period and should be given twice the normal incubation time before being discarded as nonviable.
5. For special media, growth conditions, and tips on maintenance, carefully read the literature cited in the strain descriptions. This is especially important for producers of secondary metabolites and quality control strains.

FREEZING FILAMENTOUS FUNGI

PREPARING THE CULTURE

Grow sporulating strains on solid media as for freeze-drying; grow nonsporulating strains on either solid or liquid medium. If the mycelium is easily broken, grow the culture on agar in test tubes, scrape with a pipette, and suspend the fragments in sterile 10% glycerol. Dispense 0.5 mL into each plastic vial. If the mycelium is sticky, will not break up, or grows embedded in the agar, grow the culture on agar in plates, cut out plugs containing new growth (hyphal tips) with a sterile cork borer, and place three or four plugs into each plastic freezing vial with approximately 0.4 mL of 10% glycerol.

FREEZING METHODS

To ensure long-term viability of fungal cultures, ATCC recommends freezing and storage at liquid nitrogen temperatures. Storage in the liquid itself is not always convenient or safe. Storage in liquid nitrogen vapor is a more practical alternative. It is critical, however, to constantly monitor the liquid level of the liquid nitrogen freezer to ensure that material is maintained below –130°C. Storage at warmer temperatures can compromise the stability of many strains.

1. Select a container appropriate for the material to be preserved. Plastic screw-capped vials with internal tube threads (1.0 to 2.0 mL) are appropriate for most fungi. They are sterilized by the manufacturer and, when properly handled, remain sterile throughout labeling and dispensing.
2. Label each vial clearly and accurately. Whatever labeling method is chosen, labels must be able to withstand subsequent freezing and thawing procedures.
3. Prepare the cultures for freezing as described above. Seal the plastic vials as tightly as possible with the screw cap.
4. Load the vials onto aluminum canes and record the location of each culture.
5. Cool the chamber of a controlled-rate cooling apparatus to 4°C and place the canes into the unit. Insert the thermocouple probe into one sample containing only sterile medium. Prior to starting the cooling program, allow the material to cool to within 2°C of the chamber temperature.

6 Cool the material at a rate of 1°C per minute to –40°C, then cool 10°C per minute to –90°C.

7 When the program is complete, transfer the canes to boxes in the vapor phase of a liquid nitrogen unit for storage. If the unit is more than a few feet from the programmable freezer, transport the canes in an insulated container with liquid nitrogen. Be careful not to store the vials directly in liquid nitrogen. See notes under "Safety" below. Liquid nitrogen has a temperature of –196°C. The vapor will have a temperature gradient which is near –196°C at the level of the liquid and which gradually becomes warmer near the top of the freezer. For best long-term storage, keep frozen materials below –130°C. To ensure that materials are stored at proper temperatures, liquid nitrogen freezers must first be validated by placing a thermometer at the top of the unit and adding liquid nitrogen until a working temperature of at least –130°C is maintained at the top of the freezer. This level is then continuously monitored and an alarm system activated if the levels fluctuate above or below predetermined limits. Occasionally, strains may require special handling. If a strain does not survive freezing in glycerol, try 5% DMSO. Some cultures, such as some *Agaricus* strains, may be grown on sterile seeds, grains or pollen, and may be frozen without a cryoprotectant.

RECOVERY

Thaw frozen cultures quickly in a 37°C water bath, transfer immediately to appropriate growth media, and incubate at an appropriate temperature. Given proper treatment and conditions, most cultures will grow in a few days. However, some may exhibit a prolonged lag period and should be given twice the normal incubation time before being discarded as nonviable. For special media, growth conditions, and tips on maintenance, carefully read the literature cited in the strain descriptions. This is especially important for transformation hosts, genetic mutants, producers of secondary metabolites, and quality control strains.

SAFETY

Safety precautions must be considered when preserving living cells and microorganisms by freeze-drying, freezing, and storing at cryogenic temperatures.

CRYOGENIC STORAGE

Because of its extremely cold temperature, liquid nitrogen can be hazardous if improperly used. When handling liquid nitrogen, take precautions to protect your face and exposed skin from exposure to the liquid. Wear protective clothing, including a laboratory coat, gloves designed for handling material at cryogenic temperatures, and a face shield. To reduce your exposure to cryogenic temperatures, design inventory systems for storing frozen specimens that allow for easy retrieval and that minimize the time required to look for specimens. Prolonged exposure to cryogenic temperatures can lead to a loss of sensation in the hands that can only be recovered after warming. This loss of sensation can lead to a false sense of security regarding damage to tissues by the low temperatures. When the temperature in a liquid nitrogen unit becomes tolerable and working in the unit is no longer uncomfortable, the operator has reached a point where damage from the cryogenic temperatures is likely. When liquid nitrogen is used in confined and inadequately ventilated areas, the nitrogen can quickly displace the room air. Liquid nitrogen freezers should be located in well-ventilated areas, and special precautions should be taken during fill operations. In facilities with several liquid nitrogen freezers, an oxygen monitor should be installed to warn occupants of any deterioration in the air quality due to the nitrogen gas. Plastic screw-capped vials can present a hazard if stored directly in liquid nitrogen. Vials with an inadequate seal between the cap and the vial can fill with liquid nitrogen. Upon retrieval to warmer temperatures the vials may explode violently or may spray liquid, potentially disseminating the contents of the vial. Likewise when opening plastic vials after thawing some dissemination of the contents may occur. Therefore material in plastic ampules should be stored in the vapor above the liquid nitrogen.

FREEZE-DRYING

When freeze-drying microorganisms in vials or ampules without cotton plugs or other bacteriological filters, the microorganisms can be carried from the container and contaminate the freeze-drying system. Microbial contamination can be found on the outside of the vial or ampule, and on parts of the freeze-drying system such as the condenser. A system should be designed to monitor the contamination level, and decontamination procedures should be implemented if necessary. Take care to properly treat freeze-dried cultures prior to disposal. To autoclave freeze-dried cultures, open the vial or ampule to allow penetration of the steam. An alternative to autoclaving is to heat the preparations in a hot air oven at 180°C for four hours.

CULTURE HANDLING

When opening frozen or freeze-dried cultures, take care to prevent dispersion of the ampule contents. Open these preparations in a biological safety cabinet if possible, and perform all work with hazardous cultures in a biological safety cabinet. There are varying degrees of pathogenicity among microorganisms. All laboratory personnel should be aware of the hazards posed by the cultures they are handling. Detailed discussions of laboratory safety procedures are provided in the latest U.S. Dept. of Health and Human Services / CDC publication Biosafety in Microbiological and Biomedical Laboratories. This publication is available in its entirety on the CDC Office of Health and Safety website at: www.cdc.gov/od/ohs.

CULTIVATION AND PRESERVATION LITERATURE

For any strain listed on ATCC's website, please note the recommended media and incubation conditions. The literature cited below is useful for general knowledge on the cultivation and preservation of a wide variety of fungi.

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