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Cryopreservation and Freeze-Drying of Fungi Employing Centrifugal and Shelf Freeze-Drying

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Summary

The aim of preserving a fungus is to maintain it in a viable state without change to its genetic, physiological, or anatomical characters. There are numerous methodologies available to preserve a fungus, but the two methods widely used by culture collections (biological or genetic resource centers) to achieve successful preservation are cryopreservation with liquid nitrogen using controlled-rate freezing and centrifugal freeze-drying. Generic methods are often used, but specific variations of a method may be required in order to achieve optimal stability. No single method can be applied to all fungi. More recently, techniques such as vitrification and encapsulation cryopreservation have been used to preserve recalcitrant fungi. The protocols described within this chapter have been developed over many years at one of the world's largest culture collections of filamentous fungi.

Key Words: Cryopreservation; cryoprotectant; encapsulation; fungus; lyophilization; recalcitrant; vitrification.

1. Introduction

The need for well-characterized, preserved, and authenticated fungal cultures for taxonomy, genomic, and proteomic research programs, industrial processes, conservation, testing, and education is well established ***(1)***. Cultures must retain their physiological and genetic characteristics to be suitable for their intended use. The best methodologies available to achieve this are cryopreservation and lyophilization, which can be optimized for specific species of fungi ***(1,2)***. However, the characteristics of the fungus must be monitored before, during, and after preservation procedures to ensure that preservation procedures do not compromise the integrity of the fungus ***(3)***.The main requirement

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of a preservation technique is to maintain the fungus in a viable and stable state without morphological, physiological, or genetic change until it is required for further use. Therefore, conditions of storage should be selected to minimize the risk of change. Culture preservation techniques range from continuous growth methods that reduce the rate of metabolism, to an ideal situation where metabolism is considered suspended, or nearly so ***(4,5)***. No single technique has been applied successfully to all fungi, although storage in or above liquid nitrogen is considered to be the optimum method for many fungal strains. A decision-based key is available to help researchers select the best method available for the preservation of a fungus and considers logistical, economic, and scientific criteria ***(6)***. More recently, preservation regimes have been developed for more preservation-recalcitrant fungi that otherwise could not be preserved, these techniques include encapsulation and vitrification-based cryopreservation approaches ***(1,2)***.

One factor common to all methods is the need to start with a well-characterized, healthy culture in order to obtain the best results. Optimal growth conditions including temperature, aeration, humidity, illumination, and media must be established ***(4)***. The growth requirements vary from strain to strain, although different strains of the same species and genera usually grow best on similar media. A medium that induces good sporulation and minimal mycelium formation is desirable for successful freeze-drying ***(4)***, although this is not necessary if the culture is to be cryopreserved. In some cases it is often better to grow the organism under osmotically stressful conditions, as chemicals that accumulated in the cytoplasm under these conditions may also protect the organism during cooling.

1.1. Cryopreservation

Lowering the temperature of biological material reduces the rate of metabolism until all internal water is frozen and no further biochemical reactions occur ***(7)***. This is quite often lethal and at the very least causes cellular injury. Although fungi are quite often resistant to ice-induced damage, cooling must be controlled to achieve optimum survival. The avoidance of intracellular ice and the reduction of solution, or concentration, effects are necessary ***(8)***. Little metabolic activity takes place less than –70°C. However, recrystallization of ice can occur at temperatures greater than –139°C ***(9)*** and this can cause structural damage during storage. Consequently, cooling protocols have to be carefully designed for cells in order to inflict the least damage possible. The cryopreservation of microfungi at the ultra-low temperature of –196°C in liquid nitrogen or the vapor above is currently regarded as the best method of preservation ***(1,10,11)***. It can be widely applied to sporulating and nonsporulating cultures. Initial work with fungi was undertaken by Hwang ***(12)*** who employed a method

designed for freezing avian spermatozoa ***(13)***. Similar methods have been used successfully ***(11,14,15)***. Provided adequate care is taken during freezing and thawing, the culture will not undergo any change either phenotypically or genotypically. Optimization of the technique for individual strains has enabled the preservation of organisms that have previously been recalcitrant to successful freezing ***(14)***.

The choice of cryoprotectant is a matter of experience and varies according to the organism. Glycerol gives very satisfactory results but requires time to penetrate the organism; some fungi are damaged by this prolonged incubation step. Dimethyl sulfoxide penetrates rapidly and is often more satisfactory ***(16,17)***, but is often toxic to sensitive organisms. Sugars and large molecular substances such as polyvinylpyrrolidine ***(18,19)*** have been used but, in general, have been found to be less effective.

At CAB International (CABI) (http://www.cabi-bioscience.org/bioresources.asp) over 4000 species belonging to 700 genera have been successfully frozen in 10% (v/v) glycerol. Few morphological or physiological changes have been observed in material that has been revived after successful cryopreservation ***(20)***. Finding the optimum cooling rate has been the subject of much research ***(8,12,14,15)***. Slow cooling at 1°C/min over the critical phase has proved most successful ***(15,20,21)***, but some less-sensitive isolates respond well to rapid cooling, some preferably without protectant. Slow warming may cause damage because of the recrystallization of ice, therefore, rapid thawing is recommended. Slow freezing and rapid thawing generally give high recoveries for fungi ***(22)***. Storage at –196°C in the liquid nitrogen, or at slightly higher temperatures in the vapor phase, is employed at CABI.

Occasionally, alternative techniques for the cryopreservation of recalcitrant fungi are required. Recalcitrant fungi include organisms such as *Halophythophthora*, *Saprolegnia*, and *Aphanomyces* spp., microcylic rust fungi, some Basidiomycota, and the Glomeromycota. The techniques utilized to preserve strains of these taxa include encapsulation cryopreservation and vitrification-cryopreservation techniques ***(1,23,24)***.

Vitrification of fungi ***(25)*** involves the application of very highly concentrated cryoprotectant solutions and has been applied to organisms of many cell types, especially plant cells (***24;*** *see also* Chapter 12). The technique does not require the use of controlled-rate cooling; samples are plunge cooled, with the vitrification solution forming an amorphous glass that prevents the onset of "concentration effects" or ice damage (*see* Chapters 3 and 12). On "resuscitation" from the frozen state, care must be taken to ensure that samples do not "crack," which could cause physical damage to the mycelium. Samples must be immediately washed to remove the vitrification solution, because on prolonged exposure it is toxic to the fungi. The technique has been applied to a number of

fungi with some success ***(1)***. However, vitrification solutions are extremely toxic, so routine use may not be advantageous. The use of encapsulation (immobilization) cryopreservation ***(23)***, the entrapment of mycelium/spores in calcium alginate beads prior to preservation, is well documented for fungi, for example, *Serpula lacrymans* ***(26)*** and monoxenically produced spores of *Glomeromycota* ***(27)***. Essentially, encapsulation has two main functions; it allows cells to be easily manipulated by providing a suitable suspending matrix and provides a mechanism for the water content of cells to be reduced by osmotic treatment or drying that decreases the prospects of ice damage or concentration effects during the cooling step(s) of the cryopreservation procedure. Encapsulation and vitrification cryopreservation techniques have significant potential for preserving recalcitrant fungi that could otherwise not be stored in the long term. However, the use of these techniques (for fungi) is still relatively underresearched and therefore not broadly tested.

Approaches to cryopreservation include when organisms are preserved with their host, or alternative growth substrate have been applied in various semblances for many years. The technique has potential for obligate organisms, mutualists, parasites, and yet-unculturable fungi that are otherwise not maintained in a living state by genetic resource centers. This approach has been used for recalcitrant organisms such as the microcyclic rust fungus, *Puccinia spegazzini*, where the teliospores were preserved on stem or petiole tissue ***(28)***. A similar approach incorporating encapsulation cryopreservation was used for the basidomycete fungus *Ceratobasidium cornigerum* ***(29)***, when seeds of the green-winged (*Anacamptis morio*) and common spotted (*Dactylorhiza fuchsii*) orchids were encapsulated in alginate beads with hyphae of the fungus with no adverse effects to the fungus postcryopreservation.

1.2. Freeze-Drying (Lyophilization)

Lyophilization (preservation by drying under reduced pressure from the frozen state by sublimation of ice) of fungi was first reported by Raper and Alexander ***(30)***. Improvements in methods and equipment over the years have led to a reliable and successful preservation technique for sporulating fungi ***(4)***. The technique is not suitable for preserving nonsporulating fungi, although some workers have reported limited success when lyophilizing mycelium ***(31)***. The advantages of freeze-drying over other methodologies include good stability of characters, long shelf-life, convenient storage of ampoules in the laboratory environment under ambient conditions, and easy distribution ***(22,30–32)***.

A freeze-drying system incorporates freezing the suspension, generating a vacuum, and removing the water vapor that evolved. The protectant used, rate of cooling, final temperature, rate of heat input during drying, residual moisture,

and storage conditions all affect the viability and stability of fungi ***(3,34,35)***.The suspending medium should give protection to the spores from freezing damage and during storage. Media often used are skimmed milk, serum, peptone, various sugars, or mixtures of these.

The rate of freezing is a very important factor, which must be optimized to achieve the best recovery. Slow freezing rates are employed, 1°C/min is the rate normally quoted ***(4,22)***. The technique of evaporative cooling can be used successfully for the storage of many sporulating fungi ***(4)***.

2. Materials

2.1. Liquid Nitrogen Storage

1. Biological safety cabinet and facilities for carrying out microbiological methods and aseptic techniques (*see* **Note 1**).
2. Cultures should be healthy and exhibit all characters to be preserved, both morphologically and physiologically (*see* **Note 2**).
3. Growth medium as required for cultivating different fungi ***(4)***.
4. 2 mL cryovials (System 100, Nalgene, Rochester, NY) (*see* **Note 3**).
5. Cryoprotectant: 10% (v/v) glycerol in distilled water dispensed in 10-mL aliquots, autoclaved at 121°C for 15 min.
6. Liquid nitrogen wide-necked storage tank with drawer rack inventory control system.
7. Safety equipment: should include cryogloves (cold resistant), face shield, forceps, and oxygen monitor.

2.2. Freeze-Drying

1. Biological safety cabinet and facilities for carrying out microbiological methods safely (*see* **Note 1**).
2. Cultures should be healthy and sporulating, exhibiting all characters to be preserved both morphologically and physiologically (*see* **Note 4**).
3. Growth medium as required for cultivating different fungi ***(4)***.
4. 10% (w/v) skimmed milk and 5% (w/v) inositol in distilled water dispensed in 10-mL aliquots in glass universal bottles. These are autoclaved at 114°C for 10 min (*see* **Note 5**).
5. High-voltage vacuum spark tester.
6. 0.5-mL neutral glass ampoules (Adelphi tubes, West Sussex, UK) labeled with the culture number and covered with lint caps to prevent aerial contamination (*see* **Note 6**). These are heat sterilized at 180°C for 3 h.
7. Metal racks for supporting 0.5-mL neutral glass ampoules.
8. Freeze-drier with spin freeze and manifold drying accessories.
9. Nonabsorbent cotton wool sterilized in a dry oven at 180°C for 3 h.
10. Air/gas-constricting torch.
11. Air/gas-sealing torch.
12. Heat-resistant mat.
13. Diphosphorus pentoxide general purpose reagent (GPR) (*see* **Note 7**).

2.3. Shelf Freeze-Drying (2-mL Vials)

1. 2-mL flat-bottomed preconstricted glass (ampoules) vials labeled with the culture number. These are covered with aluminium foil to prevent aerial contamination then heat sterilized in an oven at 180°C for 3 h.
2. Metal racks for holding 2-mL flat-bottomed glass vials.
3. Grooved rubber bungs sterilized by autoclaving at 121°C for 15 min then placed in 70% (v/v) industrial spirits (*see* **Note 8**).
4. Shelf freeze-dryer with programmable shelf temperature control.

3. Methods

3.1. Liquid Nitrogen Storage

All culture work should be carried out using aseptic techniques in a microbiology safety cabinet (*see* **Note 1**).

1. Grow cultures under optimal growth conditions and on suitable medium (*see* **Subheading 2.1.**, **items 2** and **3**; **Notes 2** and **9**).
2. Prepare a spore or mycelial suspension in sterile 10% (v/v) glycerol; mechanical damage must be avoided (*see* **Note 10**).
3. Add 0.5-mL aliquots of suspension to each cryovial, label with the culture number using a permanent ink cryomarker or barcode.
4. Allow at least l h for the cells to equilibrate in the glycerol (*see* **Note 11**).
5. Place the cryovials in racks and cool at approx 1°C/min in a controlled-rate cooler (*see* **Notes 12** and **13**). Alternatively, place the cryovials in the neck of the nitrogen refrigerator at –35°C for 45 min (*see* **Note 12**).
6. Transfer the cryovials into storage racks held in the vapor phase of the liquid nitrogen, this cools them to below –150°C.
7. Record the location of each culture in the inventory control system.
8. After at least 1 d retrieve an ampoule from the refrigerator to test viability and purity of the fungus.
9. Warm the cryovial rapidly by immersion in a water bath at 37°C. Remove immediately on completion of thawing and do not allow it to warm up to the temperature of the bath. Alternatively, thaw the vials in a controlled-rate cooler on a warming cycle (*see* **Note 14**).
10. Opening of the cryovial and the transfer to media should be carried out in an appropriate level microbiological safety cabinet. Surface sterilize the cryovials by immersion or wiping with 70% (v/v) alcohol. Aseptically transfer the contents using a Pasteur pipet and transfer on to suitable growth medium.

3.2. Spin Freeze-Drying

All culture work should be carried out in an appropriate microbiological safety cabinet (*see* **Note 1**).

1. Grow cultures under the optimal growth conditions for the species and on suitable media (*see* **Subheading 2.2.**, **items 2** and **3**; **Note 4**).

2. Prepare a spore suspension in sterile 10% (w/v) skimmed milk and 5% (w/v) inositol mixture.
3. Dispense 0.2-mL aliquots of the spore suspension into the sterilized and labeled ampoules ensuring the suspension does not run down the inside of the ampoule (*see* **Note 15**). Then cover the ampoules with lint caps.
4. Transfer the ampoules to the spin freeze-drier, and spin while the chamber is evacuated. Cool the suspensions at approx 10°C/min (this is uncontrolled, the actual rate depends on the amount of water and the pressure in the system).
5. After 30 min switch off the centrifuge; the spore suspension will have frozen into a wedge tapering from the base of the ampoule. This gives a greater surface area for evaporation of the liquid.
6. Dry for 3.5 h, at a pressure of between 5×10^{-2} and 8×10^{-2} mbar, then raise the chamber pressure to atmospheric pressure and remove the ampoules.
7. Plug the ampoules with a small amount of sterilized cotton wool, in a laminar air flow cabinet or a suitable microbiological safety cabinet (*see* **Note 16**).
8. Compress the plugs (aseptically) to 10 mm in depth with a glass or metal rod, and push down to just above the tip of the slope of the freeze-dried suspension.
9. Constrict the plugged ampoules using an air/gas torch just above the cotton wool plug. (The object is to ensure the glass is not drawn too thinly at the constriction and there is a sufficiently large bore left for the evacuation of air and the passage of water vapor.)
10. Hold the ampoule at each end and rotate in a narrow hot flame of the air/gas torch so it is heated evenly around an area 10 mm above the cotton wool plug; ensure that the ampoule is turned back and forth through 360°. When the glass begins to become pliable allow the flame to blow the glass in toward the center of the ampoule while rotating the ampoule slowly. Then stretch the ampoule to give no more than a 10-mm increase in total length. This is performed by moving the open-end section back and forward no more than 5 mm with equal and opposite movement of the closed end of the ampoule. When the outer diameter of the constriction is approximately one-third of its original diameter the constriction should be complete.
11. Place the ampoule down onto a heat-resistant mat while it is still slightly pliable, and roll it on the mat so the ampoule returns to a straight alignment.
12. Attach the constricted ampoules to the secondary drier and evacuate. Incubate the secondary drying stage for about 17 h, this leaves a residual moisture content of 1–2% by dry weight (*see* **Note 17**). The evolved water is absorbed by phosphorus pentoxide placed in the chamber below the manifolds of the secondary drier (*see* **Note 7**).
13. Seal the ampoules across the constriction while still attached to the manifold and under the vacuum (*see* **Note 18**) using an air/gas cross-fire burner. Support the ampoule. Project the two flames onto opposite sides of the constriction. The flames seal and subsequently cut through the glass (*see* **Note 19**). The ampoule must not be allowed to pull away from the molten seal until it has separated, or a long thin extension to the ampoule will be made. Use the flame of the torch to melt the glass top of the ampoule so it flows to form a thickened seal.

14. Before storage, test the sealed tubes with a high-voltage spark tester to ensure the seal is intact. A purple-to-blue illumination will appear inside the ampoule indicating the pressure is low enough and the seal is intact.
15. Store the ampoules under appropriate conditions (*see* **Note 20**).
16. After 2 d storage, open sample ampoules for viability and purity tests in an appropriate microbiological safety cabinet. Score the ampoule midway down the cotton wool plug with a serrated-edged, glass-cutting blade. Then heat a glass rod in a Bunsen until red hot, and press it down onto the score; the heat should crack the tube around the score.
17. Reconstitute the dried suspension and revive by adding three to four drops of sterile distilled water aseptically with a Pasteur pipet. Allow 15–20 min for absorption of the water by the spores (*see* **Note 21**).
18. Streak the contents of the ampoule onto suitable agar medium and incubate at an appropriate growth temperature.
19. It is advisable to rehydrate and check viabilities at regular intervals.

3.3. Shelf Freeze-Drying (2-mL Vials)

All culture work should be carried out in the appropriate microbiological safety cabinet (*see* **Note 1**).

1. Grow cultures under the optimal growth conditions for the species and on suitable media (*see* **Subheading 2.2.**, **items 2** and **3**; **Note 4**).
2. Prepare a spore suspension in sterile 10% (w/v) skimmed milk and 5% (w/v) inositol mixture (*see* **Note 5**).
3. Dispense 0.5-mL quantities into sterile 2-mL ampoules.
4. Aseptically insert sterile, grooved, butyl rubber bungs into the necks of the ampoules to the premolded rim so that the groove opening is above the vial lip.
5. Place the ampoules on the precooled shelf (–35°C) of the freeze-dryer (*see* **Note 22**).
6. Place the sample temperature probe into an ampoule containing the skimmed milk and inositol mixture only. When the temperature of this reaches –20°C, evacuate the chamber, this reduces the temperature of the sample to –145°C as the latent heat of evaporation is removed and rises again to the shelf temperature.
7. Maintain the shelf temperature at –35°C for 3 h and then raise to 10°C at 0.08°C/min (*see* **Note 23**).
8. After 24 h drying, from the time the temperature of the sample reaches –45°C, lower the shelf base to push the bungs into the neck of the ampoules to seal them.
9. Raise the chamber pressure to atmospheric pressure and heat seal the ampoules above the constriction using an air/gas torch ready for storage. Retain a final vacuum of approx 4×10^{-2} mbar.
10. When cool, test the sealed tubes with a high-voltage spark tester. A purple-to-blue illumination will appear inside the ampoule indicating the pressure is low enough and the seal is intact.
11. Store the ampoules in appropriate conditions (*see* **Note 20**).

12. After 4 d storage, open sample ampoules for viability and purity tests. Ampoules are snapped open at the constriction in an appropriate microbiological safety cabinet. Add 0.3 mL of sterile distilled water and then temporarily plug the ampoule with sterile cotton wool.
13. After 30 min remove the cotton wool plug and transfer the contents of the ampoule onto suitable agar medium using a Pasteur pipet. Incubate at an appropriate growth temperature.
14. It is advisable to rehydrate and check viabilities at regular intervals.

4. Notes

1. All exposure to microorganisms must be reduced to a minimum. This entails the containment of many procedures, particularly those that may create aerosols. Although good aseptic technique will contain organisms during simple transfers it is essential, where more intricate (for example, those liable to create aerosols) procedures are carried out a suitable microbiological safety cabinet is used. The latter becomes essential when hazard group 2 organisms are being handled. Hazard groups and the containment level necessary for handling them are defined in the Advisory Committee for Dangerous Pathogens, The Approved List of Biological Agents ***(33)***. The Control of Substances Hazardous to Health regulations (2002) and their subsequent amendments enforce these requirements in the United Kingdom. Fungi may also produce volatile toxins, which may be harmful to man. These too must be contained or disposed of and a suitable cabinet should be used. A class II microbiological safety cabinet is recommended as this not only protects the worker, but also protects the cultures from contamination. Good practice should ensure that appropriate containment facilities are also used for hazard group 1 organisms. Fungi of the higher hazard group 3 require total containment in a class III microbiological cabinet or use of a glove box.
2. Sterile cultures survive the technique well but it is often best to allow full development before preservation. Sporulating cultures give better recovery. Poor isolates will not be improved by this method and may be more sensitive to the process, giving rise to preservation failures.
3. Unless stated by the manufacturer, plastic cryovials should only be stored in the vapor phase to avoid seepage of nitrogen through the cap seal.
4. Only sporulating fungi seem to survive centrifugal freeze-drying, though some sterile ascocarps, sclerotia, and other resting stages have been processed successfully. However, the method of shelf freeze-drying (*see* Chapter 8) can be much more successful for these than the spin-freeze method. Cooling rate, drying temperature, and rate of heat input can be optimized for the organism. Organisms have survived this method, which have failed spin freeze-drying ***(34)***.
5. The skimmed milk and inositol mixture is sensitive to heat and denatures easily. The sugars are caramelized, therefore the temperature and the time of exposure to high temperatures must be controlled. Autoclave at 114°C for 10 min.
6. Lint caps (fluffy side innermost) can be made to go over individual tubes or batches of tubes. At CABI, 15 replicate ampoules are covered by one cap. The tubes are placed in metal racks covered with aluminium foil and sterilized.

7. The phosphorus pentoxide desiccant is harmful and caustic, all contact must be avoided. Desiccant trays should be filled carefully to avoid bringing the powder into the air. Goggles, gloves, and particle masks should be worn.
8. Sterilization of the butyl rubber bungs by autoclaving at 121°C for 15 min will introduce water into the bung that will be liberated during the freeze-drying process or storage afterwards. Immersion in sterile industrial methylated spirits will help remove the water and can be evaporated away. Alternatively, surface sterilization with industrial methylated spirits without autoclaving can be sufficient to prevent contamination of the freeze-dried product.
9. Cold hardening of the cultures prior to freezing may prove beneficial. Pregrowth of cultures in the refrigerator (4–7°C) can improve post-thaw viabilities of some fungi, though others cannot grow at low temperatures. For those isolates that are sensitive, a short exposure to these temperatures may be tolerated, or this stage can be omitted altogether.
10. Various precautions can be taken to prevent mechanical damage. Fungi on slivers, or blocks, of agar can be placed in the ampoules. Alternatively, the fungus can be grown on small amounts of agar in the ampoule before the cryoprotectant is added (plastic cryotubes are more suitable for this). An alternative is to grow cultures on plant seeds in liquid culture or on small inanimate particles. These can then be transferred to the cryotube and frozen.
11. Cryoprotectants protect in several different ways but they must be allowed to come to equilibrium with the cells. The permeable protectants must be given time to enter the cell and this time depends on the permeability of the cell membrane. Generally the cell membrane of fungi is more permeable to dimethyl sulfoxide than to glycerol. Larger molecular weight substances, such as sugars and polyvinylpyrrolidone, do not penetrate the cell and either protect by reducing the amount of water in the cell through exosmosis, or impede ice crystal formation. Normally a period of at least 1 h is necessary for equilibration (*see* Chapter 3).
12. Liquid nitrogen is considered to be a hazardous substance; being extremely cold it will produce injuries similar to burns and it is also an asphyxiant gas at room temperature. It is important that it is handled with care. Contact must be avoided with the liquid or anything it has come into contact with. A face shield should be worn to prevent splashes hitting the face. Cold-resistant gloves will prevent direct contact but care must be taken not to allow nitrogen to splash into them.

    Liquid nitrogen can penetrate incompletely sealed vials during storage. On retrieval of the vial from liquid nitrogen the liquid will expand and, if it unable to escape, may cause the ampoule to explode.

    The liquid nitrogen and culture storage vessels must be stored in a well-ventilated area, ideally with low-level extractor fans installed. It is recommended that the level of oxygen in the area be monitored. If the level of oxygen in the atmosphere falls below 18% (v/v) anyone present will suffer drowsiness and headache. If the level falls to 16% (v/v) this is potentially lethal. As the generated nitrogen is initially cold there will be a higher concentration closer to the ground. If a person faints they would be in danger of asphyxiation. At CABI the liquid nitrogen storage

area has a low-level oxygen alarm and in other areas where liquid nitrogen is used staff are issued with an individual oxygen monitor.

13. A reproducible method of cooling to the holding temperature can be achieved in a controlled-rate cooler. CABI uses a Kryo 16 programmable cooler (Planer Products Ltd., Sunburg, UK). The control temperature is measured in the chamber wall and therefore the sample temperature can vary quite widely from the programmed protocol. The program must take this into account and be adjusted until linear cooling of the sample is achieved.
14. A controlled-rate cooler can be programmed to thaw the frozen fungi. The ampoules are heated at about 200°C/min raising the chamber temperature to 50°C. The sample is removed before its temperature reaches 20°C.
15. When filling the ampoules it is important not to allow the suspension to run down the length of its inner surface. When the ampoule is heated during constriction, the suspension burns releasing fumes that may be toxic to the freeze-dried material and leaves residues that could interfere with the eventual sealing of the ampoule.
16. The period when ampoules are kept at atmospheric pressure between drying stages must be as short as possible, as the exposure to the atmosphere of the partly dried material can cause deterioration ***(35)***.
17. Excessive drying will be lethal to cells, or at least may induce mutation by damaging the DNA ***(36)***. Also, having too high a residual moisture will result in rapid deterioration during storage ***(34)***. A residual water content of between 1 and 2% by dry weight proves successful for fungi.
18. At CABI evacuation continues while sealing after the second stage drying to ensure low pressure levels in the ampoule and therefore good storage conditions. An alternative method is to back fill with a dry inert gas such as nitrogen or argon.
19. If the flame is allowed to heat the ampoule on either side of the constriction during sealing, the molten glass will be pushed in by atmospheric pressure and the ampoule may implode.
20. Storing the ampoules at a low temperature is thought to give greater longevities, and 4°C seems to be favored by many workers in the field ***(34)***. At CABI the ampoules are stored at temperatures between 15 and 20°C and fungi have survived over 30 yr ***(4)***.
21. Rehydration of the fungi should be carried out slowly giving time for the absorption of moisture before plating onto a suitable medium. It is sometimes necessary to rehydrate preserved specimens in a controlled environment for some very sensitive strains ***(37)***.
22. The shelf temperature of a shelf freeze-drier can be controlled to cool at a particular rate. This enables the cooling stage of the freeze-drying process to be optimized for individual fungal strains. However, it may be preferable to precool the ampoules in a programmable cooler and then transfer them onto precooled shelves.
23. The temperature of the sample must be kept below its melting point during drying. The shelf temperature is therefore kept low (–35°C) during the initial stages of the process. The freezing point of fungal cytoplasm is quite often between –15

and –20°C ***(34)*** and, therefore, the temperature must remain below this until all unbound water is removed. The suspension reaches 5% moisture content after 3 h, and the warming protocol takes over 6 h to rise to –20°C.

References

1. Ryan, M. J. and Smith, D. (2004) Fungal Genetic Resource Centres and the genomic challenge. *Mycol. Res*. **108,** 1351–1362.
2. Smith, D. and Ryan, M. J. (2004) Current status of fungal collections and their role in biotechnology. In: *Handbook of Fungal Biotech*nology, *2nd ed.*, (Arora, D. K., ed.), Marcel Dekker, Inc., New York, pp. 527–538.
3. Ryan, M. J., Bridge, P. D., Smith, D., and Jeffries, P. (2002) Phenotypic degeneration occurs during sector formation in *Metarhizium anisopliae*. *J. Appl. Microbiol.* **93,** 163–168.
4. Smith, D. and Onions, A. H. S. (1994) *The Preservation and Maintenance of Living Fung, 2nd ed*. CAB International, Wallingford, UK.
5. Calcott, P. H. (1978) *Freezing and Thawing Microbes*. Meadowfield Press, Durham, UK.
6. Ryan, M. J., Smith, D., and Jeffries, P. (2000) A decision-based key to determine the most appropriate protocol for the preservation of fungi. *WJMB* **16,** 183–186.
7. Franks, F. (1981) Biophysics and biochemistry of low temperatures and freezing. In: *Effects of Low Temperature of Biological Membranes,* (Morris, G. J. and Clarke A., eds.), Academic Press, London, UK, pp. 3–19.
8. Smith, D. (1993) Tolerance to freezing and thawing. In: *Stress Tolerance of Fungi,* (Jennings, D.H., ed.), Marcel Dekker, Inc., New York, pp. 145–171.
9. Morris, G. J. (1981) *Cryopreservation: An Introduction to Cryopreservation in Culture Collections. Culture Centre of Algae and Protozoa.* ITE, Cambridge, UK.
10. Kirsop, B. E. and Doyle, A. (1991) *Maintenance of Microorganisms and Cultured Cells: A Manual of Laboratory Methods.* Academic Press, London, UK.
11. Smith, D., Ryan, M. J., and Day, J. G. (2001) *The UKNCC Biological Resource: Properties, Maintenance, and Management*. UKNCC Secretariat, Egham, Surrey, UK.
12. Hwang, S. -W. (1960) Effects of ultralow temperature on the viability of selected fungus strains. *Mycologia* **52,** 527–529.
13. Polge, C., Smith, A. U., and Parkes, S. (1949) Revival of spermatozoa after dehydration at low temperatures. *Nature* London **164,** 666.
14. Morris, G. J., Smith, D., and Coulson, G. E. (1988) A comparative study of the morphology of hyphae during freezing with the viability upon thawing of 20 species of fungi. *J. Gen. Microbiol.* **134,** 2897–2906.
15. Smith, D. and Thomas, V. E. (1998) Cryogenic light microscopy and the development of cooling protocols for the cryopreservation of filamentous fungi. *WJMB* **14,** 49–57.
16. Hwang, S. -W. and Howell, A. (1968) Investigation of ultra-low temperature of fungal cultures II. Cryoprotection afforded by glycerol and dimethyl sulphoxide to eight selected fungal cultures. *Mycologia* **60,** 622–626.

17. Hwang, S. -W., Kylix, W. F., and Haynes, W. C. (1976) Investigation of ultra low temperature for fungal cultures III. Viability and growth rate of mycelial cultures cryogenic storage. *Mycologia* **68,** 377–387.
18. Ashwood-Smith, M. J. and Warby, C. (1971) Studies on the molecular weight and cryoprotective properties of PVP and Dextran. *Cryobiology* **8,** 453–464.
19. Smith, D. (1983) Cryoprotectants and the cryopreservation of fungi. *Trans. Br. Mycol. Soc*. **80,** 360–363.
20. Hwang, S. -W. (1966) Long term preservation of fungus cultures with liquid nitrogen refrigeration. *Appl. Microbiol*. **14,** 784–788.
21. Hwang, S. -W. (1968) Investigation of ultra low temperature for fungal cultures I. An evaluation of liquid nitrogen storage for preservation of selected fungal cultures. *Mycologia* **60,** 613–621.
22. Heckly, R. J. (1978) Preservation of microorganisms. *Adv. Appl. Microbiol*. **24,** 1–53.
23. Benson, E. E., Wilkinson, M., Todd, A., Ekuere, U., and Lyon, J. (1996) Developmental competence and ploidy stability in plants regenerated from cryopreserved potato shoot tips. *CryoLetters* **17,** 119–128.
24. Benson, E. E. (1994) Cryopreservation. In: P*ractical Plant Cell Culture: A Practical Approach,* (Dixon, R. A., ed.), IRL Press, Oxford, UK, pp. 147–166.
25. Tan, C. S. and Stalpers, J. A. (1996) Vitrification of fungi. In: *Biodiversity, International Biodiversity Seminar ECCO XIV Meeting,* (Cimerman, A. and Gunde-Cimerman, N., eds.), ECCO, Ljubljana, Slovenia, pp. 189–193.
26. Ryan, M. J. (2001) The use of immobilisation for the preservation of *Serpula lacrymans*. *Mycologist* **15,** 66–69.
27. Declerck, S. and Coppenolle, M. G. A. V. (2000) Cryopreservation of entrapped monoxenically produced spores of an arbuscular mycorrhizal fungus. *New Phytot*. **148,** 169–176.
28. Ryan, M. J. and Ellison, C. (2003) Development of a cryopreservation protocol for the microcyclic rust-fungus *Puccinia Spegazzinii*. *CryoLetters* **24,** 43–48.
29. Wood, C. B., Pritchard, H. W., and Miller, A. P. (2000) Simultaneous preservation of orchid seed and its fungal symbiont using encapsulation-dehydration is dependent on moisture content and storage temperature. *CryoLetters* **21,** 125–136.
30. Raper, K. B. and Alexander, D. F. (1945) Preservation of moulds by the lyophil process. *Mycologia* **37,** 499–525.
31. Tan, C. S. (1997) Preservation of fungi. *Cryptogamic Mycology* **18,** 157–163.
32. von Arx, J. A. and Schipper, M. A. A. (1978) The CBS fungus collection. *Adv. Appl. Microbiol*. **24,** 215–236.
33. Advisory Committee for Dangerous Pathogens (2004) *The Approved List of Biological Agents.* HSE London, HMSO Norwich, UK.
34. Smith, D. (1986) *The evaluation and development of techniques for the preservation of living fungi.* PhD Thesis, University of London, London, UK.
35. Smith, D. and Kolkowski, J. (1992) Fungi. In: *Preservation and Maintenance of cultures used in Biotechnology and Industry*. Butterworth Publishing Company, Stoneham, MA.

36. Rey, L. R. (1977) Glimpses into the fundamental aspects of freeze drying. In: *Development in Biological Standardisation. International Symposium of Freeze Drying of Biological Products,* (Cabasso, V. J., Regamey R. H., and Krager S., eds.), Basel, Switzerland, pp. 19–27.
37. Ashwood-Smith, M. J. and Grant, E. (1976) Mutation induction in bacteria by freeze-drying. *Cryobiology* **13,** 206–213.
38. Staffeld, E. E. and Sharp, E. L. (1954) Modified lyophil method for preservation of *Pythium* species. *Phytopathology* **44,** 213–214.