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Freeze-Drying Fungi Using a Shelf Freeze-Drier

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Summary

Lyophilization, the removal of water by freezing and then volatilization at low pressure and temperature, has been employed as a standard long-term preservation method for many filamentous fungi. The method outlined involves the use of standard shelf freeze-drying and skimmed milk as a suspending solution/lyoprotectant. This approach has been employed to freeze-dry the majority of the 50,000 fungal strains that have been successfully lyophilized at the Centraal bureau voor Schimmelcultures (CBS) culture collection (http://www.cbs.knaw.nl/).

Key Words: Freeze-drying; fungi; lyophilization.

1. Introduction

Lyophilization is the preferred method for cultures that must be dispatched because, unlike cryopreserved cultures, these cultures do not need to be revived prior to dispatch. In the cooling step preceding drying, the extracellular water crystallizes, consequently the cells dehydrate and, surrounded by the highly viscose lyoprotectant, they become embedded in a feathered pattern of the ice crystals. During primary drying the temperature is reduced to such extent that the lyoprotectant becomes so viscose that it transforms into a glass. A glass is a liquid in which the molecules are immobilized ***(1)***. In the primary drying phase the ice crystals are evaporated, leaving a glass interwoven with channels. By slowly increasing the temperature to that of the secondary drying phase, water is sublimated from this glass and escapes through these channels, making the glass even more viscose. Finally, at the end of the secondary drying phase, when only 1–2% (w/v) moisture is left, the suspending medium is so viscose that it is in the glass phase at room temperature. As in cryopreservation, a glass

From: *Methods in Molecular Biology, vol. 368: Cryopreservation and Freeze-Drying Protocols, Second Edition*
Edited by: J. G. Day and G. N. Stacey © Humana Press Inc., Totowa, NJ

is an ideal formulation in which to store the dehydrated organisms (*see* Chapters 2 and 3), because there is no enzyme or chemical activity and the molecules are arranged in an unordered structure, allowing protection of the membranes and the proteins ***(2)***.

Skimmed milk is an ideal lyoprotectant, which is cheap (also standard skimmed milk, available from any foodstore, may be employed). The glass transition temperature (Tg) of frozen skimmed milk is –18°C, which facilitates freeze-drying and contains a mixture of macromolecules (lactalbumine and casein) and a saccharide. The macromolecules serve as bulking agents, the amino acids contained in the macromolecules help to repair sublethal damage and provide the energy during revival. The saccharide, lactose, helps to diminish transitions of the membrane during dehydration by replacing the dipoles of the water ***(3–5)***. Furthermore, denaturation of the proteins during cooling is diminished by saccharides ***(6)***. They repel the hydrophobic parts of the amino acids, thus preventing unfolding of the proteins (preferential exclusion) ***(7–9)***. To improve survival, 7% (w/v) trehalose can be added to the skimmed milk. This disaccharide "fits best" within the membrane structure ***(10)*** and produces a stable glass at a relatively high Tg value ***(11)***. Trehalose is actually produced in the spores and conidia of fungi and yeasts to protect their membranes, and proteins at the low water activity Aw values present in these propagules ***(12,13)***. Survival levels might also be increased by adding 1% (w/v) Na-glutamate to the lyoprotectant. Like the saccharides, amino acids diminish protein denaturation during freeze-drying by the mechanism of preferential exclusion.

2. Materials

1. Biological safety cabinet and facilities for carrying out microbiological methods safely.
2. Cultures should be healthy and sporulating, exhibiting all characters to be preserved both morphologically and physiologically.
3. Growth medium as required for cultivating different fungi.
4. A lyoprotectant of 12% (w/v) Tyndalized skimmed milk (*see* **Note 1**).
5. Cooling system, for example, an insulated container with a mixture of solid carbon dioxide and acetone, or alternatively a controlled-rate cooler.
6. 0.5-mL neutral borosilicate glass ampoules. These are heat sterilized at 180°C for 3 h.
7. Metal racks for supporting 0.5 mL glass ampoules.
8. Shelf freeze-drier.
9. Nonabsorbent cotton wool sterilized at 180°C for 3 h.
10. Air/gas-constricting torch.
11. Air/gas-sealing torch.
12. Heat-resistant mat.

3. Methods

3.1. Preparation Suspension in Lyoprotectant

All culture transfers should be carried out using aseptic techniques in an appropriate microbiology safety cabinet for the organisms to be preserved. It is important that all local safety requirements are adhered to.

1. Make a spore or conidia suspension in 12% (w/v) Tyndalized skimmed milk (*see* **Note 1**).
2. Add 50–200 µL spore suspension, depending on the concentration to a borosilicate freeze-drying ampoule (*see* **Note 2**).
3. Close the ampoule with a cotton plug.
4. Cool the propagule suspension to at least –45°C (*see* **Note 3**).

3.2. Freeze-Drying

To avoid "collapse," the whole freeze-drying process must be performed below the Tg. Tg is the temperature at which a glass melts during warming. When the temperature is raised above the Tg, the frozen suspension melts and is liquid- instead of freeze-dried resulting in a tremendous protein denaturation. Tg can be estimated with the aid of a differential scanning calorimeter ***(14)***. Alternatively, most manufacturers of freeze-drying devices recommend the use of a eutectic monitor to establish the temperature of complete solidification (eutectic temperature) (*see* **Note 4**).

1. Load the freeze-drying device at a shelf temperature of –35°C to avoid melting during loading.
2. Dry suspension in the primary drying phase at least 5°C below Tg of the frozen suspension (*see* **Note 1**).
3. Consult a phase transition diagram to establish the vacuum of the primary drying phase (*see* **Note 5**).
4. Choose the vacuum that corresponds with a product temperature of 10°C below Tg (*see* **Table 1**) (e.g., skimmed milk: 0.470 mbar; skimmed milk + 7% (w/v) trehalose: 0.220 mbar; skimmed milk + 7% (w/v) trehalose + 1% (w/v) Na-glutamate: 0.140 mbar (*see* **Table 2**) (*see* **Note 5**).
5. Choose a safety vacuum that corresponds with the Tg temperature (*see* **Note 1**) (e.g., skimmed milk: 0.1.250 mbar; skimmed milk + 7% [w/v] trehalose: 0.630 mbar; skimmed milk + 7% [w/v] trehalose + 1% [w/v] Na-glutamate: 0.420 mbar [*see* **Note 5**]).
6. The end of the primary drying phase is detected by a pressure rise test. In this procedure the valve between the condenser and the drying chamber is closed. A pressure increase after closure of the valve indicates that not all the frozen water is evaporated. When a pressure rise test cannot be performed, primary drying should last approx 16 h when the thickness of the layer of material to be dried is 1–2 mm (*see* **Note 6**).

Table 1
Eutectic Temperature, Glass-Transition Temperature, and Temperature Primary Drying Phase of Various Lyoprotectants

Lyoprotectant	Teut (°C)	Tg (°C)	T prim. dr. phase (°C)
12% (w/v) sm	–16	–18	–23
12% (w/v) sm + 7% (w/v) tr	–17	–25	–30
12% (w/v) sm + 7% (w/v) tr + 1% (w/v) Na-gl	–24	–29	–34

Na-gl, Na-glutamate; sm, skim milk; Teut, eutectic temperature; Tg, glass-transition temperature; T prim. Dr. phase, primary drying phase; tr, trehalose.

7. Increase temperature to 25°C to reach the secondary drying phase. Increase the temperature slowly (maximum rate 1°C/min) to avoid collapse (*see* **Note 7**).
8. Apply maximum vacuum during the secondary drying.
9. Continue secondary drying until a residual moisture content of 1–2% is reached. This will take 6–9 h (*see* **Note 8**).
10. Close the ampoules after secondary drying, while still being under vacuum (*see* **Note 9**).
11. Heat-seal glass ampoules while still being under vacuum to avoid leakage during storage. To guarantee axenicity during opening, the ampoules should be sealed above the cotton plug.
12. Store freeze-dried ampoules at 4°C (*see* **Note 10**).

3.3. Revival

1. Open the ampoule just above the cotton plug by scoring the glass wall with a glass cutter or a sharp file, and break it at the scored mark.
2. Flame the opening and remove the cotton plug with sterile forceps. Suspend the material by pouring it into a tube containing 1–2 mL sterile water (*see* **Note 11**).
3. Pour the suspension over a suitable agar medium and incubate at optimal conditions.

4. Notes

1. Optionally 7% (w/v) trehalose and/or 1% (w/v) Na-glutamate can be added to the 12% (w/v) skimmed milk to improve survival rates, however, Na-glutamate decreases Tg substantially (*see* **Table 1**). Consequently, when this component is added to the protectant, low temperatures must be applied during drying and increased drying periods. When a new protocol is developed, estimate Tg of the dried product by differential scanning calorimeter to establish the storage temperature.
2. This glass quality is needed because the ampoules must be heat-sealed after drying.
3. Cool one-celled (<4 µm), thin-walled propagules, e.g., those from *Aspergillus* ssp., *Penicillium* ssp., *Trichoderma* ssp., *Acremonium* ssp., *Verticillium* ssp. instantaneously to avoid protein denaturation. Propagules can be cooled instantaneously by immersing them in a mixture of solid carbon dioxide and acetone (–75°C). Cool septate fungi (>4 µm in diameter), e.g., *Alternaria* ssp., *Curvularia* ssp., *Fusarium* ssp.,

Table 2
Phase-Transition Data Showing Relation Between Pressure Above Ice and Temperature of Ice

°C	mbar	°C	mbar	°C	mbar	°C	mbar
0	6.110	–20	1.030	–40	0.120	–60	0.011
–1	5.620	–21	0.940	–41	0.110	–61	0.009
–2	5.170	–22	0.850	–42	0.100	–62	0.008
–3	4.760	–23	0.770	–43	0.090	–63	0.007
–4	4.370	–24	0.700	–44	0.080	–64	0.006
–5	4.020	–25	0.630	–45	0.070	–65	0.0054
–6	3.690	–26	0.570	–46	0.060	–66	0.0047
–7	3.380	–27	0.520	–47	0.055	–67	0.0041
–8	3.010	–28	0.470	–48	0.050	–68	0.0035
–9	2.840	–29	0.420	–49	0.045	–69	0.0030
–10	2.560	–30	0.370	–50	0.040	–70	0.0026
–11	2.380	–31	0.340	–51	0.035	–71	0.0023
–12	2.170	–32	0.310	–52	0.030	–72	0.0019
–13	1.980	–33	0.280	–53	0.025	–73	0.0017
–14	1.810	–34	0.250	–54	0.024	–74	0.0014
–15	1.650	–35	0.220	–55	0.021	–75	0.0012
–16	1.510	–36	0.200	–56	0.018	–76	0.0010
–17	1.370	–37	0.180	–57	0.016	–77	
–18	1.250	–38	0.160	–58	0.014	–78	
–19	1.140	–39	0.140	–59	0.012	–79	

and one-celled thick-walled (*Periconia* ssp.) propagules slowly at –1°C/min to avoid production of intracellular ice crystals ***(15)*** in a controlled-rate cooler.

4. In this measurement, a drop in electrical resistance of the product indicates when the solid phase changes to liquid. However, when some of the components of the lyoprotectant, such as the disaccharide trehalose solidified as a glass instead of a eutectic, eutectic temperature deviates substantially from Tg (*see* **Table 1**).
5. In a phase-transition diagram, the relation between the temperature of ice and the pressure above this ice is shown (*see* **Table 2**).
6. When 1% (w/v) Na-glutamate is added, primary drying should be at least 48 h to dry a layer with thickness of 1–2 mm.
7. When 1% (w/v) Na-glutamate is added to the protectant the temperature should be increased to 25°C at a maximum rate of 0.5°C/h.
8. When 1% (w/v) Na-glutamate is added to the protectant, secondary drying at 30°C instead of 25°C is recommended, and the secondary drying phase is prolonged to 16 h (layer thickness, 2 mm).
9. Dried membranes, proteins, and even DNA are very vulnerable to damage caused by free radicals. Moreover, the half-life of free radicals is increased in dehydrated material.
10. Check viability immediately after drying (to check whether the organisms survive the freeze-drying process), after 5-yr storage, or 1-wk storage at 37°C to check stability of the dried product.
11. The suspension can be poured as soon as the pellet is dissolved. However, to improve the recovery process, it is advised to incubate the suspension 16 h at 20–24°C. Alternatively 2 mL malt-peptone solution can be used to restore sublethal damage caused to the membranes and proteins by freezing and drying, and to restore the energy charge.

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