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Freeze-Drying of Yeast Cultures

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Summary

A method is described that allows yeast species to be stored using a variation on the standard freeze-drying method, which employs evaporative cooling in a two-stage process. Yeast cultures are placed in glass ampoules after having been mixed with a lyoprotectant. Primary drying is carried out using a centrifuge head connected to a standard freeze-dryer. Once the centrifuge head is running, air is removed and evaporated liquid is captured in the freeze-dryer. Centrifugation continues for 15 min and primary drying for a further 3 h. The ampoules are constricted using a glass blowing torch. They are then placed on the freeze-dryer manifold for secondary drying under vacuum overnight, using phosphorus pentoxide as a desiccant. The ampoules are sealed and removed from the manifold by melting the constricted section.

Although the process causes an initial large drop in viability, further losses after storage are minimal. Yeast strains have remained viable for more than 30 yr when stored using this method and sufficient cells are recovered to produce new working stocks. Although survival rates are strain specific, nearly all National Collection of Yeast Cultures strains covering most yeast genera, have been successfully stored with little or no detectable change in strain characteristics.

Key Words: Yeast; lyophilization; freeze-drying; lyoprotectant; trehalose; skimmed milk; horse serum; ampoules; evaporative cooling.

1. Introduction

Freeze-drying is a method that is commonly used to preserve a wide variety of microorganisms and has been long used to preserve yeast cultures ***(1–3)***. The term "freeze-drying" generally refers to the technique of the removal of water by sublimation of a frozen culture sample under vacuum (*see* Chapter 2). The method used by the National Collection of Yeast Cultures (NCYC) (http://www.ncyc.co.uk/) is more correctly described as "air-drying" as it dispenses with the initial freezing of the samples to be stored and relies on evaporative cooling by the application of a vacuum during the primary drying process to remove water from the sample.

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

The removed water vapor is trapped and the dried material is stored under vacuum in ampoules sealed after the secondary drying stage.

Although viability levels are not as high as those seen in cultures stored under liquid nitrogen, the product has the advantages of only requiring low maintenance, protecting the cultures from contamination during storage, and providing an easy method for distribution of cultures by mail that is particularly valuable to culture collections. The NCYC has successfully stored a wide variety of yeast species by this method and has found that after the initial drying process viability levels tend to remain static, and cultures are recoverable after periods greater than 30 yr. Furthermore, changes in the characteristics of yeast stored are either very minor or undetectable.

2. Materials

1. Growth medium: YM broth/agar. Dissolve 3 g each yeast and malt extracts, 5 g peptone, and 10 g glucose in 1 L of distilled water (pH not adjusted). Autoclave for 15 min at 121°C and dispense aseptically into suitable bottles for storage. Final pH should be approx 6.2. Alternatively, dissolve 21 g of Becton Dickinson Franklin Lakes, NJ. YM Broth (271120) in glass distilled water and make up to 1 L. Autoclave and dispense as above. Agar (Oxoid agar no. 1, OXOID Ltd. Basingstoke, Hampshire, UK), when required, is added to a final concentration of 1.5–2% (w/v).
2. Lyoprotectant/suspending medium (*see* **Note 1**).
   a. Horse serum + glucose: dissolve 7.5 g glucose in 80 mL horse serum (heat inactivated, mycoplasma screened, analytical grade) and make up to 100 mL. Filter-sterilize using 0.22-µm filters and dispense into sterile 1-oz plastic-capped bottles. Store at 4°C.
   b. Skimmed milk + trehalose + monosodium glutamate (sodium glutamate): dissolve 10 g skimmed milk, 10 g trehalose, and 5 g sodium glutamate (all food grade) in 80 mL distilled water and make up to 100 mL. Dispense into 1-oz universal bottles and sterilize by autoclaving for 5 min at 121°C.
3. Incubator: temperature controlled at 25°C.
4. Centrifuge: suitable for 1-oz bottles containing approx 10 mL YM broth culture, capable of approx 2500 rpm (900*g*) for up to 10 min.
5. Ampoules: 0.5 mL neutral glass ampoules for freeze-drying, approx 8-mm outer diameter and 10-cm long. Wash before use with detergent and rinse with demineralized water.
6. Cotton wool: nonabsorbent, white.
7. Filter paper: Whatman no. 1 or similar.
8. Freeze-dryer: freeze-dryer with centrifuge, cooling chamber, and manifold (Edwards Whatman: Florham Park, NJ; BOC/Edwards Wilmington, MA, Modulyo or similar model).
9. Glass blowing torch: butane/oxygen torch with single tip suitable for constriction of ampoules.

10. Ampoule sealing torch: butane/propane hand-held blowtorch suitable for producing a small directional flame for sealing and removing ampoules from freeze-dryer manifold.
11. Dessicant: phosphorus pentoxide.
12. High-frequency spark tester.
13. Sapphire or metal glass cutter/scorer.
14. Fume cupboard.
15. Ampoule storage area: preferably temperature controlled at 1°C.

3. Methods

1. Preparation of ampoules: Labels for the ampoules are prepared from strips of Whatman No. 1 filter paper or similar. The number of the yeast strain and the date of the freeze-drying are printed on the label, either with a stamp using nontoxic ink or with a laser printer (*see* **Note 2**). The label is folded in half lengthwise and inserted into the ampoule so that the writing faces outward. The ampoule is loosely plugged with nonabsorbent cotton-wool and placed in a tin or suitable container with a lid for oven sterilization. Ampoules for different yeast strains should be placed in separate tins. Oven-sterilize for 4 h at 180°C.
2. Preparation of inoculum: The culture to be freeze-dried is grown without aeration (*see* **Note 3**) in YM broth at 25°C for 72 h; the amount needed depends on the number of ampoules to be prepared (*see* **Note 4**).
3. Lyoprotectant/suspending medium. Use either horse serum + glucose as detailed in **Subheading 2.**, **item 2a**, or skimmed milk + trehalose + monosodium glutamate as detailed in **Subheading 2.**, **item 2b** (*see* **Note 5**).
4. Filling of ampoules. The bottles containing the yeast culture and YM broth should be centrifuged for approx 10 min at 2500 rpm (900*g*) to ensure that the yeast culture has formed a pellet in the bottom of each bottle. Once removed from the centrifuge, all the liquid should be aseptically removed from one of the duplicate bottles and approximately half the liquid from the other, taking care not to disturb the pellet. The yeast culture pellet in the bottle with the liquid should then be resuspended in the liquid and transferred aseptically to the other bottle, where it is mixed with the second pellet to give a cell suspension double the concentration of the original culture in a single bottle. Equal volumes of this suspension and the lyoprotectant are then mixed aseptically in a separate sterile bottle; the volume prepared depending on the number of ampoules to be freeze-dried.

   A sterile 30-dropper Pasteur pipet is used to transfer six drops (approx 0.15 mL) of the mixture to each ampoule, after which the cotton-wool plug is replaced. Care should be taken not to touch the insides of the ampoule with the pipet so that each of the drops from the pipet reaches the bottom of the tube and is not allowed to run down the inside of the ampoule (*see* **Note 6**). The open plug end of the ampoule is briefly flamed to sterilize it before the cotton-wool is replaced. The remaining lyoprotectant/culture mixture is retained for viability estimation. If this is not to be done immediately, the culture should be cooled in a refrigerator to slow further cell division.

Fig. 1. Centrifuge head with filled ampoules awaiting primary drying.

5. Primary drying. The filled ampoules are placed immediately in the centrifuge head of the freeze-dryer with the writing on the labels facing the center of the centrifuge head so as not to be obscured by the dried yeast after centrifugation (*see* **Fig. 1**). The centrifuge head is placed on the spindle in the drying chamber. The refrigeration mechanism of the freeze-dryer should be switched on sufficiently early to allow the temperature of the vapor collection chamber in the freeze-dryer to be lowered to –50°C before primary drying begins (*see* **Note 7**).

   The centrifuge is switched on 10 s before starting the vacuum pump, allowing any droplets of the mixture still on the inside of the ampoule to be forced to the bottom of the tube before drying commences. Centrifugation should continue for 10 min. Primary drying is continued for 3 h, after which time the vacuum is released and the centrifuge head and ampoules are removed from the drier. The yeast in the ampoules should appear completely dry and should have formed a slope of suspended material against the wall in each of the ampoules.
6. Ampoule constriction. Trim the projecting ends of the cotton-wool plugs with scissors that have been sterilized by flaming with ethanol. The remainder of each plug is then pushed into the ampoule with a plunger that has also been sterilized by flaming with ethanol (*see* **Note 8**). The plug should be pushed sufficiently far into the ampoule so that the flame does not scorch it during the constriction process.

   Using the butane/oxygen glass blowing torch, constrict the ampoules at a point above the level of the plug. Once heated, the glass wall of the ampoule should melt forming a thicker walled central section to the ampoule with a narrow

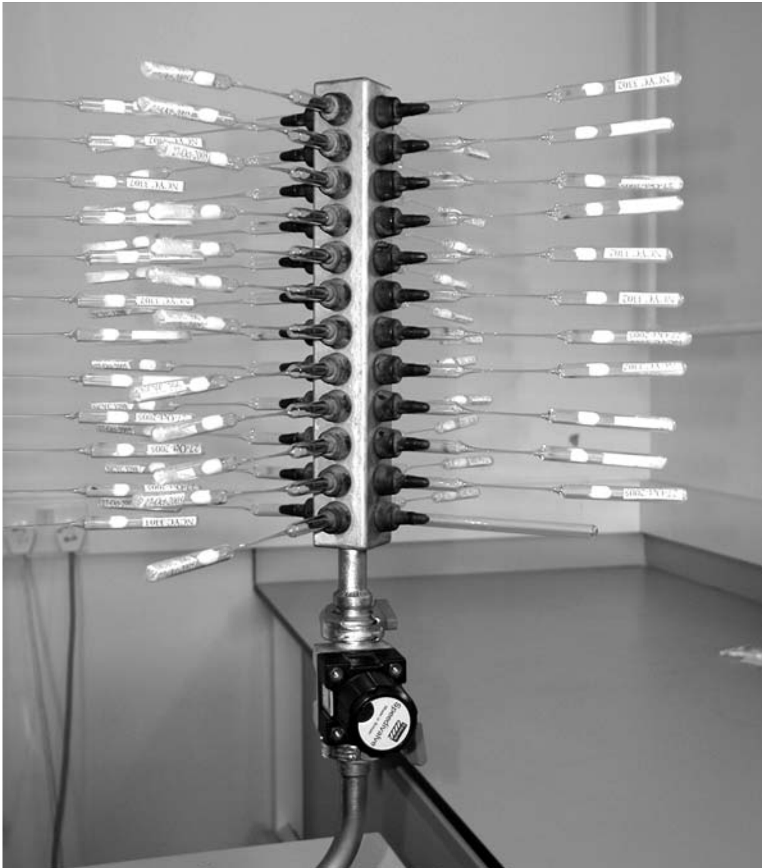

Fig. 2. Constricted ampoules on manifold during secondary drying.

central bore. The ampoule should be constantly rotated as it is heated to keep the heating even and to keep the two ends of the ampoule in alignment. The central constricted section of the ampoule should be thick enough to support the weight of the end section when it is placed on the manifold for secondary drying (*see* **Note 9**).

7. Secondary drying. Ensure that the vapor collection chamber is completely dry, fill the desiccant trays with phosphorus pentoxide, and place them in the chamber (*see* **Note 10**). Place the manifold over the chamber and place the constricted ampoules on the manifold taking care not to damage the manifold nipples or crack the necks of the ampoules. Any unused spaces on the manifold can be filled with unconstricted ampoules. Once all the spaces have been filled the vacuum pump can be started. For secondary drying the bleed valve on the vacuum pump should be shut.

   Secondary drying is normally continued overnight, although it may be terminated after 2 h, allowing the whole process to be completed in a single day.

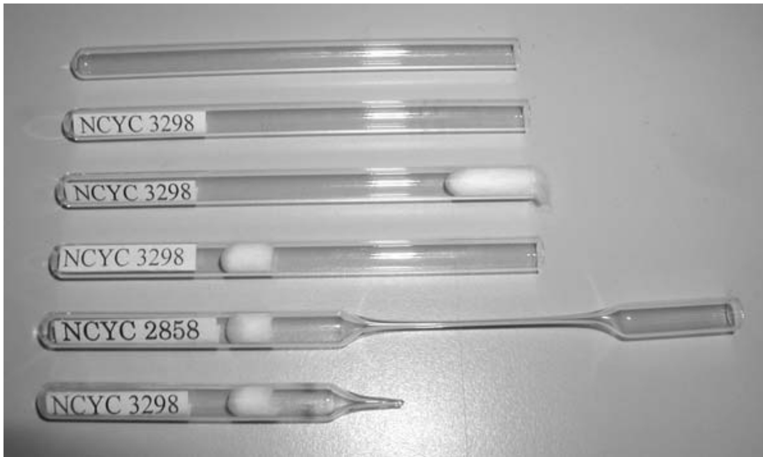

Fig. 3.

8. Removal of ampoules from the manifold. The ampoules can be sealed and removed with the use of a small blowtorch. The flame should be applied to the constricted part of the ampoule while the end containing the dried yeast is supported to prevent it drooping. The central bore of the constriction should close, seal, and then separate from the end attached to the manifold if the end of the ampoule is slowly and gently pulled away from the manifold. Any sharp spikes, either on the detached ampoule or the end still attached to the manifold, should be rounded off by melting with the blowtorch.

   Once all the ampoules have been removed the vacuum pump can be switched off and the chamber valve opened. Spent phosphorus pentoxide should be covered with water in a fume cupboard and then discarded (*see* **Note 11**). The remaining part of the ampoules, still attached to the manifold, should be removed, placed in a "discard-pot" for sterilization by autoclaving, and subsequently discarded.
9. Testing. The ampoules should be tested to make sure they contain a vacuum by using a high-frequency spark tester. The end of the spark tester should be briefly touched on the surface of each ampoule. Ampoules containing a vacuum will glow blue/violet when the spark is applied. Ampoules containing air will not glow and should be discarded (*see* **Note 12**).
10. Quality control and viability counts. On cultures prior to drying: add 1 mL of the mixed lyoprotectant/yeast suspension to 9 mL of sterile glass-distilled water. Prepare further logarithmic dilutions to 10^{-6}. Transfer three drops from a 30-dropper pipet (0.1 mL) of dilutions 10^{-6} to 10^{-3} onto YM agar. Incubate the plates at 25°C for 72 h or longer, if necessary (*see* **Note 13**). One or two drops from the mixture are also placed on an agar plate and streaked across the surface as per standard "streak-plate" procedure. The streak plate is incubated with the serial dilution plate and should be used to check for the presence of contaminants, aberrant colonies or any other anomalies when compared with the postfreeze-drying streak plate.

On cultures after freeze-drying. The count is carried out as soon as possible after freeze-drying. Open the ampoule as outlined (*see* **Note 14**). An aliquot (1 mL) of YM broth is transferred to a sterile bijoux bottle (7 mL or one-fourth ounce bottles). Using a Pasteur pipet the entire contents of the bijoux are transferred aseptically to the opened ampoule. The yeast is thoroughly resuspended and mixed with the pipet contents, taking care not to allow the YM broth to overflow the opened ampoule. One or two drops of the resuspended yeast are also placed on an agar plate and streaked across the surface as per standard "streak-plate" procedure, incubated as previously mentioned, and used as a comparison with the predried culture. All the remaining suspension is returned to the bijoux. This suspension is a 10^{-1} dilution. Prepare further logarithmic dilutions to 10^{-5}. Transfer three drops from a 30-dropper pipet (0.1 mL) of dilutions 10^{-5} to 10^{-2} onto YM agar. Incubate the plates at 25°C for 72 h, or longer if necessary.

The number of colonies formed by each dilution of pre- and postdrying suspensions is counted and the viability of the culture is calculated and recorded. Any ampoules without vacuum, containing mixed or contaminated cultures, or those that have low viability should be discarded.

11. Storage of ampoules. Storing ampoules at low temperatures (between 1 and 4°C) in the dark is believed to prevent deterioration of the dried yeast (*see* **Note 15**).

4. Notes

1. Several different substances have been shown to be effective as lyoprotectants and have significantly increased the survival rates of freeze-dried cultures. The NCYC successfully used a mixture of horse serum and glucose for many years, but now uses a mixture based on skimmed milk and other food-grade ingredients similar to that described by Berny and Hennebert ***(4)*** (*see* **Note 5**). If only a few yeast strains are to be maintained it may be possible to establish the optimum lyoprotectant for each strain. The NCYC has chosen the two lyoprotectants listed as ones that have worked well with a wide range of strains covering most yeast genera and giving acceptable survival rates.
2. Ampoules may be labeled on the outside using a labeling machine. However, the NCYC prefers to use internal labels as these cannot become separated from the ampoule, or be rendered unreadable without destroying the ampoule. Furthermore, studies performed at the NCYC have suggested that the viability of cultures originating from ampoules with internal labels was higher that that of those with no filter paper inside. This indicates that the presence of the filter paper may have a beneficial effect during the drying process.
3. The NCYC has observed that the degree of oxygenation provided during growth of the culture may affect survival during drying. Although response to oxygen is strain specific, in general, higher survival levels are obtained following growth in nonoxygenated media ***(3)***. It was also noted that cultures of sensitive strain grown on nutritionally poor medium before freeze-drying had higher survival levels.
4. Standard NCYC procedure is to inoculate two 1-oz bottles each containing 10 mL YM broth for each set of 16 ampoules to be filled. The bottles and media should be of equal weight so as to be balanced when centrifuged.

5. Both lyoprotectants listed have been used successfully over many years by the NCYC. Because of considerations of health and safety, as well as customer acceptability, it is standard NCYC policy to use the skimmed milk-based lyoprotectant with any cultures that are likely to be used as ingredients for, or in preparation of, any beverages or foodstuffs, as the ingredients can all be found as "food-grade" products.
6. When the ampoule is heated during constriction, plug any of the dried yeast suspension on the inside of the glass above the level of the will burn, releasing fumes that may lower viability and blacken the inside of the ampoule. A significant percentage of the yeast culture can be lost in this way.
7. The freeze-dryer vents should be shut in preparation to produce the vacuum, although it may be preferable to allow a small amount of air to flow through the vacuum pump bleed valve in order to let air from the atmosphere pass through the oil to reduce the amount of dissolved vapor retained in it.
8. The plunger should be flamed and sterilized when moving between batches of ampoules containing different strains, so as to prevent cross-contamination.
9. Ultraviolet-proof tinted safety glasses should be worn during the constriction process and appropriate care taken to prevent burns when using the glass blowing torch.
10. Phosphorus pentoxide desiccant is harmful and can cause burns on skin contact. Desiccant trays should be filled in a fume cupboard to avoid inhalation of the powder, and appropriate safety clothing and goggles should be worn.
11. Phosphorus pentoxide reacts vigorously with water. This operation only should be performed in a fume cupboard and appropriate safety clothing and goggles should be worn.
12. The spark tester can cause electric shock and burns. Care should be taken not to bring the electrical discharge from the tester into contact with flesh. There is some evidence to suggest that prolonged exposure to the electrical discharge can lower the viability of the yeast in the ampoules. The amount of time the spark tester is in contact with the ampoules should be kept as short as possible. Carrying out the testing away from bright light sources can make the "glow" in the ampoules easier to see.
13. The plates are kept horizontal at all times to ensure the drops remain discrete. Dilutions containing 20–30 colonies are used for estimating viability. The number of cells per milliliter inoculated into the ampoule is equal to the number of colonies in three drops multiplied by 10 times the dilution factor.
14. Score the ampoule with an ampoule cutter or file just above the level of the cotton-wool plug (toward the pointed end of the ampoule). Sterilize the ampoule with 70% (v/v) ethanol. Apply the end of a red-hot glass rod to the file mark to crack the glass. Remove the tip of the ampoule and extract the plug with sterile forceps and place them in a container for subsequent sterilization before disposal.
15. NCYC cultures are stored in the dark at 1°C. The NCYC holds strains that are still viable 30 yr after being freeze-dried. Other laboratories hold strains that have proven viable after longer periods though it has been noted that occasionally strains suddenly lose viability ***(3)***. If possible viability should be monitored on a regular basis.

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