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Cryopreservation of Yeast Cultures

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Summary

A method is described that allows a wide range of yeast species to be stored in liquid nitrogen while maintaining a high level of viability. Yeast cultures are sealed in commercially available polypropylene straws after having been mixed with a glycerol-based cryoprotectant. Once placed in a secondary cryotube the temperature of the sealed straws is reduced slowly to –30°C in a methanol bath over a period of up to 3 h. The straws are then transferred directly to the liquid nitrogen and placed in a racking system for long-term storage.

Key Words: Yeast; cryopreservation; liquid nitrogen; cryoprotectant; glycerol; straws; dehydration.

1. Introduction

Yeast cultures are held in long-term storage in the National Collection of Yeast Cultures (NCYC) (http://www.ncyc.co.uk/) by two methods: (1) freeze-dried in glass ampoules, and (2) under liquid nitrogen using glycerol as a cryoprotectant. Freeze-drying is a generally accepted method for yeast storage, having the advantages of conferring longevity and genetic stability, as well as being suitable for easy worldwide postal distribution of the cultures in glass ampoules. However, preservation by freeze-drying tends to be much more labor intensive than storage in liquid nitrogen and requires a higher level of skill to produce an acceptable product. Strain viabilities are generally low, typically being between 1 and 30%, as compared with more than 30% for those of yeast preserved by being frozen in liquid nitrogen. There are also several yeast genera, including *Lipomyces, Leucosporidium*, and *Rhodosporidium* that have particularly low survival levels and frequently cannot be successfully freeze-dried by the standard method.

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However, some improvements were made in the 1990s using trehalose as a protectant ***(1,2)*** and these were incorporated into NCYC procedures. A technique used by the NCYC for freeze-drying yeasts can be found in Chapter 6 of this volume.

Storage of cultures in liquid nitrogen, although technically simple, can involve relatively high running costs because of the necessity of regular filling of the containers. The initial cost of the equipment is comparable to that used for freeze-dying, but the costs and problems associated with the handling of liquid nitrogen have led some collections to seek alternatives ***(3)***. However, for most workers, the technique of liquid nitrogen storage is convenient, well tried and tested, and unlikely to be superseded in the near future.

The method presented here uses heat-sealed straws and is a miniaturized version of that commonly used. This enables a considerable reduction in storage space and extra protection against contamination by liquid nitrogen leakage. Storage in straws was first described in 1978 by Gilmour et al. ***(4)*** using artificial insemination straws and variations on the original method are now in use in a large number of laboratories around the world. Work on refining methods of storage in liquid nitrogen is continuing, as is research into the effects of the freezing process on the cells. The following paragraphs give an outline of the current understanding.

During the process of liquid nitrogen storage, certain changes take place in the cells and their immediate environment ***(5)***. As the straws are cooled, extracellular ice formation results in an increase in the solute concentration around the cells causing them to lose water and shrink ***(6,7)*** (*see* Chapter 3 for a review). This freeze-induced dehydration causes the cell wall to decrease in surface area and increase in thickness. As the maximum packing density of the lipids in the cell membrane bilayer is reached its normal structure changes. Membrane invaginations occur to allow the cells to shrink further as water is removed. This process is reversible, provided that none of the membrane material becomes lost within the cytoplasm, and upon thawing, the cell will return to its normal volume.

Cell shrinkage during freezing is vital to prevent cellular damage, hence the need to select the correct cooling rate. If the cooling rate is too rapid there is insufficient time for the cells to lose water and intracellular ice formation then occurs that causes damage to cell organelles ***(7)***.

Genetic damage may occur if the nucleus becomes disrupted. Plasmids have been found to be lost from strains of bacteria by the freezing and thawing processes ***(8)***. Similar loss of plasmids has been observed in *Saccharomyces cerevisiae*, along with mitochondrial damage that results in respiratory-deficient cells giving rise to petite colonies ***(5)***.

2. Materials

The procedures described involve use of potentially hazardous materials. The relevant local safety regulations (e.g., COSHH regulations in the UK) should be consulted prior to implementation of these procedures.

1. 21 g/L YM broth (Becton Dickinson/DIFCO Franklin Lakes, NJ): Difco dehydrated YM broth (cat. no. 271120).
   Alternatively:

YM media	Per liter
Yeast extract	3 g
Malt extract	3 g
Peptone	5 g
Glucose	10 g

   After mixing the pH should be between 5.0 and 6.0. Dispense in 10-mL volumes into suitable bottles. Sterilize by autoclaving for 15 min at 121°C.
2. YM agar: add 2% (w/v) agar to Difco YM broth or YM media before sterilization. After mixing the pH should be between 5.0 and 6.0. Sterilize by autoclaving for 15 min at 121°C. Dispense in 20-mL aliquots into sterile Petri dishes and leave to cool.
3. 10% (v/v) glycerol cryoprotectant solution: dissolve 10 mL glycerol in 90 mL distilled water. Sterilize by filtration through a 0.22-µm filter and dispense in 15-mL aliquots into suitable bottles. Store at room temperature.
4. Straws: cut colored polypropylene drinking straws to 2.5-cm lengths (*see* **Note 1**). Seal one end of each straw by holding firmly with nonridged forceps 2 mm from the end to be sealed, bringing the projecting end l cm from the flame of a Bunsen burner (*see* **Fig. 1**; **Note 2**). Once the polypropylene has melted sufficiently to close the end of the straw (usually within 1–2 s) the straw should be removed from the heat and allowed to cool while still firmly held with the forceps. Once the polypropylene has solidified, the straw can be released.

   Place the straws in a glass Petri dish and sterilize by autoclaving at 121°C for 15 min. For ease of handling, the straws should be evenly spaced around the edge of the dish with all the open ends pointing in the same direction. Two long, unsealed straws should also be prepared for use as rests for straws awaiting final sealing (*see* **Fig. 2**; **Note 3**). These should also be placed in glass Petri dishes and sterilized. Ensure that the straws are dry before use, using a moderate-temperature (40–60°C) drying cabinet if necessary.
5. Nunc cryotubes: 1.8-mL plastic screw-cap ampoules are available sterilized from the manufacturer (Nunc International, Rochester, NY; Fisher Scientific UK, Loughborough, Leicester, UK; VWR International Ltd., West Chester, PA).
6. A refrigerated methanol bath precooled to –30°C (*see* **Note 4**).
7. Liquid nitrogen containers: cryogenic storage containers with liquid-phase storage racks and dividers to store 2-mL cryotubes (e.g., Jencons Scientific Ltd.) (*see* **Fig. 3**).
8. Safety equipment: cryogloves, goggles, and so on (e.g., Jencons Scientific Ltd., East Grinstead, West Sussex, UK).

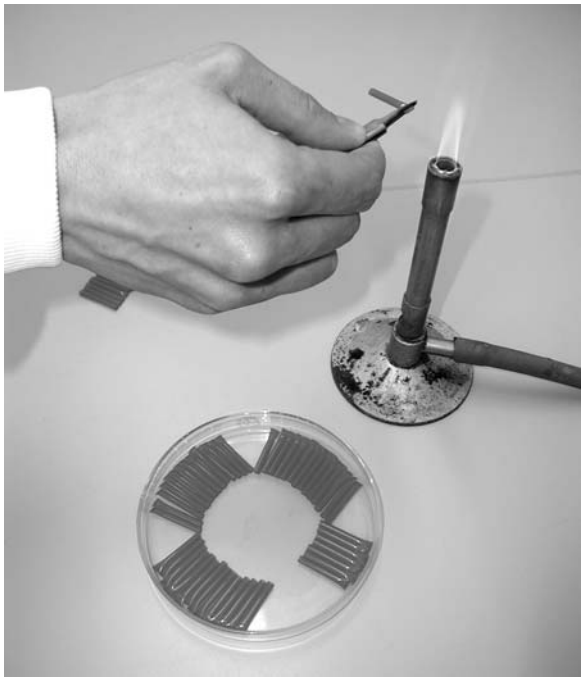

Fig. 1. Sealing polypropylene straws.

Fig. 2. Filled polypropylene straws sealed at one end awaiting final sealing and (lower center) sealed straws in a cryotube awaiting transfer to methanol bath.

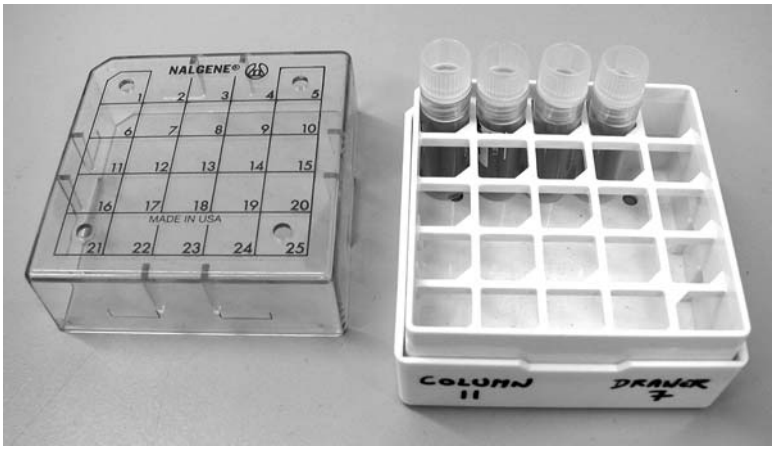

Fig. 3. Filled cryotubes in numbered racking system.

3. Methods

Follow good microbiological practice and aseptic techniques throughout.

1. Grow the culture to be frozen in 10 mL of YM broth for 72 h at 25°C on a reciprocal shaker.
2. Mix equal amounts of inoculum and glycerol cryoprotectant solution aseptically in a sterile bottle (*see* **Note 5**). Use forceps, sterilized by immersion in alcohol followed by flaming, to remove a single straw from the Petri dish. Gently grip the straw about halfway along its length to allow the insertion of the end of a Pasteur pipet containing the inoculum. Insert the pipet until the pipet tip is at the sealed end of the straw and then withdraw it as the inoculum fills the straw. Fill the straw to approximately two-thirds of its capacity. On withdrawing the pipet from the straw, any excess inoculum can be sucked back into the pipet (*see* **Notes 6** and **7**).
3. The open end of the straw is clamped shut with the forceps and sealed as described (*see* **Subheading 2.**, **item 4**). Care should be taken not to allow any of the contents of the straw to be forced out of the open end of the straw as it is clamped with the forceps, as this will prevent a good seal from being made.
4. Test the straws for leaks by holding them with forceps above the surface of a suitable disinfectant in a high-sided beaker and then squeezing them. Any liquid forced out of the seals should be safely contained within the beaker. Discard leaking straws and autoclave them.
5. Place six straws in each 1.8-mL cryotube. If post-thaw cell viability counts are required, a single straw is placed in a separate ampoule for ease of recovery. Mark each straw and cryotube with the relevant strain designation and date of freezing using a black Pentel (Pentel Co. Ltd., Swindon, Wiltshire, UK) permanent marker pen.
6. Place the filled cryotubes in the methanol bath, which has been precooled to –30°C, for 2 h to allow dehydration (*see* **Note 8**).

7. Transfer the cooled cryotubes to the liquid nitrogen containers (*see* **Note 9**) and place in the racking (inventory) system (*see* **Notes 10** and **11**). Note the position of the cryotubes (*see* **Note 12**). Remove excess methanol from the outside of the cryotubes to prevent it freezing the tubes to the racking system once immersed in the liquid nitrogen (*see* **Notes 9**, **13**, and **14**).
8. After storage: locate the cryotube and remove it from the racking system, check the strain number and date of freezing written on the tube. Remove a single straw and replace the cryotube in the racking system (*see* **Note 15**). Rapidly transfer the straw to a water bath, incubate at 35°C, and agitate to ensure that rapid and even thawing takes place (*see* **Note 16**).
9. Remove the straw from the water bath and dry it. Grip one end of the straw and sterilize the other end by wiping with 70% (v/v) alcohol. Cut off the sterile end with scissors that have been flamed with alcohol. Then remove the contents using a Pasteur pipet inserted into the open mouth of the straw. Mix the contents by repeated pipetting before transferring as an inoculum to suitable growth media (*see* **Note 17**).
10. Viability counts.
    a. On cultures prior to freezing: add 1 mL of the original cell 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 18**).
    b. On cultures after freezing: add two drops from a Pasteur pipet of the thawed cell suspension (0.06 mL) to 0.54 mL of sterile, glass-distilled water. Transfer 0.5 mL of this 10^{-1} dilution to 4.5 mL of sterile, glass-distilled water, prepare additional logarithmic dilutions to 10^{-6} as detailed previously, then inoculate YM agar plates, incubate, and count as in **step 10**. The percentage viability of the culture is calculated and recorded.

4. Notes

1. The NCYC uses polypropylene drinking straws in preference to insemination straws because of their low cost, robust nature, and ability to withstand autoclaving. Straws of approx 3- to 4-mm diameter were found to be the most suitable to use with this method.
2. Polypropylene straws should melt quickly and will form a strong seal that will set firm within 3–4 s of being removed from the heat. Care should be taken not to burn the polypropylene or deform the rest of the straw. A standard Bunsen burner may be used although "fishtail" will give a more controllable flame. Occasionally, leaks occur at the corners of the straw; particular attention should be paid to these areas to ensure they are properly sealed. Some workers have used impulse heat sealers to seal polypropylene straws. In our experience these tend to produce weak seals and do not produce the characteristic "lip" formed by the melted polypropylene that is useful when removing straws from cryotubes.
3. If several straws are being filled with the same inoculum it is convenient to transfer each straw to a sterile Petri dish before it is sealed. The straws should be leaned with the open end against a second longer straw to allow their easy removal for sealing and to prevent leaking from the opening (*see* **Fig. 2**). The straws are then

sealed and placed in the cryotube. This is more efficient than filling and sealing each straw separately.

4. **Caution**: methanol is toxic and flammable. Avoid skin contact.
5. Other workers have successfully used cryoprotectants other than glycerol. Some have been used with a wide range of yeasts, others with single strains only. Substances used include 10% (v/v) each of dimethyl sulfoxide ***(4)***, ethanol ***(9)***, methanol ***(10)***, YM broth, and 5% (w/v) hydroxyethyl starch.
6. Care should be taken not to allow droplets to remain on the open end of the straw because these can prevent proper sealing.
7. The final cell concentration in the straws once the inoculum and cryoprotectant are mixed is between 10^6 and 10^7 cells/mL. Final glycerol concentration in the straws is 5% (v/v).
8. Experiments on two test strains of *S. cerevisiae* at the NCYC showed no significant variation in viability for cells frozen to –20, –30, or –40°C for 1, 2, or 3 h during primary freezing. The NCYC uses the intermediate range that has so far been successful for all NCYC strains. A study carried out by Pearson et al. ***(5)*** indicated that cooling rates greater than 8°C/min result in a sharp drop in cell viability and cause irreversible genetic damage. Cells have been found to be more sensitive to cooling rates than glycerol concentration ***(5)***. Survival of cells appears to be related to the avoidance of intracellular ice formation and excessive cryodehydration ***(7)***.
9. If there is a large distance between the methanol bath and liquid nitrogen containers, a precooled Dewar should be used to transport the tube to prevent warming.
10. Care should be taken to provide adequate ventilation where liquid nitrogen is in use as a buildup of nitrogen can cause asphyxiation. Workers should not travel in lifts with containers of liquid nitrogen. Goggles and gloves should be worn while carrying out operations using liquid nitrogen.
11. Several models of liquid nitrogen storage container are available. Some offer greater amounts of storage space by reducing the level of liquid nitrogen above the racking system to a minimum. In order to ensure that the top racks of these containers are always completely submerged, they must be filled more regularly than those with less height of racking. These containers are best avoided unless an automatic "top-up" system is available.
12. Because stock levels cannot be easily checked once immersed in liquid nitrogen, accurate record keeping of both stock levels and the position of each strain in the racking system is important. Computerized stock control systems are ideal for storage of this information. The position of each cryotube in a rack may be mapped and recorded in the following way, for example:

Strain number:	240
LN_2 container:	A
Section:	3
Tray:	2
Row:	1
Position:	6
Total no. of straws:	6

If only a small number of strains are to be stored, different colored caps can be used for color coding of cryotubes.

13. Cultures can be stored successfully in the vapor phase of liquid nitrogen. However, because changes may still occur in the stored cells at temperatures greater than –139°C, storage in the liquid phase at –196°C is preferable. While the cultures are submerged in the liquid nitrogen temperature stability is guaranteed. If storage is only required for short periods higher temperatures may be adequate.
14. The long-term survival of yeasts held in liquid nitrogen has not been well documented, but all evidence suggests that losses during storage are insignificant. The NCYC has found no drop in viability of cultures stored for up to 10 yr. Work with mutant strains of *S. cerevisiae* and *Schizosaccharomyces pombe* at the NCYC has also demonstrated that genetic stability is also very good.
15. If the racking system used holds many cryotubes they will be exposed to higher temperatures when the racking system is removed from the liquid nitrogen to recover a straw. Care should be taken to minimize this time as much as possible. Work done at the NCYC has suggested that using straws sealed inside cryotubes offers considerable protection against short exposure to higher temperatures. No significant drop in viability has been recorded in straws held in a racking system that has been repeatedly removed briefly from the liquid nitrogen.
16. Nunc recommends that cryotubes should not be used for freezing in liquid nitrogen unless correctly sealed in Cryoflex because trapped nitrogen can expand and cause the tubes to explode once they are removed from the liquid. Because, in this method, the cryotube is only being used for secondary containment, the screw cap should not be firmly tightened. This will allow trapped nitrogen to leave the tube safely as its temperature increases. However, tubes should always be held at arm's length when being removed and safety goggles and gloves should be worn. Very occasionally straws will rupture on thawing from poor seals letting in liquid nitrogen. Provision should be made for sterilizing benchtops and equipment if they become contaminated with the contents of the ruptured straw.
17. Cells removed from liquid nitrogen storage should be transferred initially to small aliquots (10 mL) of suitable growth media. Larger volumes of media can be inoculated from this culture once sufficient growth has occurred.
18. 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 straw is equal to the number of colonies in three drops multiplied by 10 times the dilution factor.

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