

CRYOPRESERVATION OF YEASTS IN POLYPROPYLENE STRAWS

J. Henry and B. Kirsop

National Collection of Yeast Cultures, AFRC Institute of Food Research
Norwich Laboratory
Colney Lane, Norwich NR4 7UA
United Kingdom

Introduction

It has been shown (1,2) that the survival levels of yeasts stored in liquid nitrogen at -196°C are high and superior to those obtained with other preservation methods. Good strain stability following cryopreservation is also recorded. Storage at temperatures -130°C below which molecular activity does not occur, can be achieved by storage in the liquid phase of nitrogen or in mechanical refrigerators operating at these temperatures (3) and are to be preferred to storage at high temperatures. The method described here is for storage in liquid nitrogen. Cultures may be stored in polypropylene cryotubes, glass vials or polypropylene straws. This method uses straws and has the following advantages:

- Economies of space (5-6 straws can be stored in each cryotube).
- Prevention of seepage of liquid nitrogen into the samples; liquid nitrogen may penetrate the washers of polypropylene cryotubes, but cannot penetrate the sealed straws.
- Polypropylene drinking straws are cheap and readily obtainable throughout the world.
- Polypropylene is very much safer to use than glass vials.
- The straws are available in different colours, allowing colour-coding of samples.
- The culture can be recovered for use by removal of a single straw, the rest of the samples remaining frozen.

Method

1. Preparation of straws

Coloured polypropylene straws (available from most catering distributors and many shops) are cut into 2.5 cm lengths. One end of a straw is sealed by holding firmly in a pair of unridged forceps 1 mm inwards so that the projecting end is 1 cm from the flame of a fish-tail bunsen burner. The polypropylene melts almost immediately and forms a strong seal that sets firm within a second or two.

The straws are placed in a glass petri dish and autoclaved at 121°C for 15 min.

2. Inoculum

The culture to be frozen is grown in YM broth (Difco Laboratories Ltd. 0711-01) at 25°C for 72 h, if possible on a reciprocal shaker. Each straw requires 0.1 ml of a suspension containing between 10^6 and 10^7 cells ml⁻¹. Cell concentration has been found to have little effect on the percentage of cells surviving.

3. Preparation of cryoprotectant

A 10% glycerol solution is prepared, filter-sterilised, and stored in sterile screw-cap bottles.

4. Inoculation of straws

Equal quantities of inoculum and cryoprotectant are mixed aseptically in a sterile bottle. A single straw is removed with forceps from the petri dish and filled with inoculum using a Pasteur pipette. When filling it is necessary to place the end of the pipette close to the sealed end of the straw and to fill to within 3 mm of the open end. The filled straw is then sealed at the open end as described in step 1. above.

Six straws are placed in each plastic, screw-capped 2 ml ampoule (Nunc, Gibco-Europe Ltd).

5. Freezing

Primary freezing. It is very important to wear protective clothing when using liquid nitrogen refrigerators or handling frozen specimens. Ampoules are frozen to -30°C by placing in a refrigerated room. If a room at -30°C is not available, a refrigerated cabinet or cooling bath (Camlab Ltd) may be used. If aluminium canes (Union Carbide) are to be used for the secondary freezing, ampoules may be placed on the canes at this stage. If secondary freezing is to take place in storage drawers (Union Carbide), the ampoules may be well spaced in wire racks for the primary freezing. The cooling rate is not critical in this method, but is probably in the region of 5°C min⁻¹, depending on the size of samples and containers. Cells are held at -30°C for 2 h to allow dehydration to take place.

Secondary freezing. The ampoules are transferred to the canisters or storage drawers of the refrigerator (Union Carbide) and immersed in the liquid nitrogen, care being taken to prevent the samples from thawing. If the distance between primary and secondary freezing containers is great, samples should be transported in a chilled Dewar or in any other suitable container.

6. Revival

Using sterile forceps, a straw is removed from the ampoule and placed immediately into a screw-cap bottle containing water at 35 C. The bottle is shaken to facilitate rapid thawing. Several straws may be thawed simultaneously in this way.

7. Viable counts

Before opening the straws, the cells are redispersed by squeezing the straws carefully several times. The straws are then wiped with 95% alcohol and one end is cut off using sterile scissors. The contents are removed using a Pasteur pipette. It may be necessary to disperse cells further by repeated pipetting at this stage. Two drops of the suspension (0.06ml) are transferred to 0.54ml sterile water to make a 10^{-1} dilution. Further dilutions, plating and counting are carried out using standard methods (4).

8. Storage

Ampoules are stored in the liquid nitrogen refrigerator (Union Carbide), care being taken to maintain the liquid nitrogen at such a level that ampoules are completely submerged.

Notes

1. A number of other cryoprotectants have been used successfully both by the NCYC and other workers. Some have been used for a fairly wide range of yeasts, others with a few strains only. Substances used successfully include glycerol (20%, 10%, 5%), dimethyl sulphoxide (DMSO, 10%), glycerol plus DMSO, ethanol (10%), methanol (10%), YM broth and hydroxyethyl starch (10%, 5%).
2. The NCYC has found that primary freezing and dehydration at -20 C, -30 C or -40 C for 1, 2 or 3 h is equally successful for the two test strains of *Saccharomyces cerevisiae* used to develop the method. The intermediate protocol (-30 C for 2 h) has been adopted and proved successful for a wide range of species.
3. The NCYC has found that, in general, higher levels of survival are obtained from aerobically grown cultures than from those grown with

limited access to oxygen.

4. It is important that the outside of the straws is kept dry during filling, as wet straws do not seal well.
5. When filling several straws with the same inoculum, it is convenient to lay each straw as it is filled against a glass rod in a sterile petri dish until all straws are filled. The straws are then sealed and put into ampoules. This is more convenient than filling and sealing each straw separately.
6. If the outsides of the straws are dry, they do not adhere to each other when placed in ampoules. Removal of straws from ampoules is facilitated if straws vary slightly in length.
7. The different coloured straws can be used to colour-code yeast strains to aid retrieval from the refrigerators, and it is clearly sensible to store only one yeast strain in each ampoule.

Survival levels and shelf life

Survival levels between 50 and 100% are frequently obtained for a wide range of yeast species, using the standard method. Higher survival levels for individual strains could probably be obtained by careful adjustments to the protocol. The NCYC has detected no drop in viability in test strains over a period of 26 months and others record good survival for periods of up to 4 years. In view of the high initial survival rates, shelf life can be expected to be good.

References

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