

CRYOPRESERVATION OF FUNGI

P. Hoffmann, Ph.D.

DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
Mascheroder Weg 1B, D-3300 Braunschweig
Federal Republic of Germany

Introduction

Cryopreservation in liquid nitrogen (LN) is a reliable method for long term storage of microorganisms. Different protocols have been published (Kirsop & Snell, 1984; Smith & Onions, 1983; Elliott, 1976; Stalpers et al., 1987), varying with equipment, special needs, preference of materials or the type of organism. A simple method is described here, which is applicable to a large variety of microorganisms such as mycelial fungi, molds and yeasts.

Materials and Methods

Equipment

Different types of storage tanks which accommodate 1.0 to 1.8 ml cryotubes (e.g. Intermed NUNC, Denmark) may be employed. The "canister & cane system" (e.g. COSMOS L40, Messer Griessheim, F.R. Germany) (Fig. 1 I, K), if available, is preferred because it allows the removal of only one "cane", holding several ampoules at a time (Fig. 1 I). The storage capacity of tanks employing the "drawer system" is greater (e.g. BT 55, Air Liquide, France), however, LN evaporation rates are higher and removal of one ampoule requires lifting of a whole set of drawers out of the storage container.

PVC (polyvinylchloride) or polypropylene "straws" with a diameter of 2-3 mm are cut to a length of approx. 25 mm on a paper cutting machine. One end of the PVC straws is immersed into acetone for a few seconds and is heat sealed at a temperature of approximately 280 C; other materials may be sealed in a gas

flame. The sealed straws are sterilized by autoclaving (15 min, 121 C). PVC straws not treated with acetone usually reopen during sterilization.

For sealing of the straws a commercial household sealing machine with adjustable temperature may be used.

Forceps with specially designed tips will greatly facilitate handling of the straws.

A block accommodating the frozen cryotubes is recommended, if opening of the tubes outside of the container is necessary and thawing of remaining straws is to be avoided. The block is either made of aluminum, brass or copper and is surrounded by a styrofoam carrying case. It will keep the temperature of the cryotubes below -120° for about 15 min.

Preparation of organisms

A schematic outline of the procedure is given in Fig. 1.. Yeasts are grown in liquid culture (Fig. 1 B) or on a suitable solid medium (Fig.1 A) to a colony size of approximately 2mm Ø . Two colonies of the strain are removed from the agar with a loop, carefully suspended in 1.5 ml sterile glycerol (10% w/v in water) (Fig. 1 C) and filled into the sterile straws (sealed at one end) with a disposable syringe or Pasteur pipette (Fig. 1 D). The straws may then be sealed completely and transferred aseptically to a sterile cryotube (Fig. 1 H).

Sporulating fungi are grown on solid media until conidia develop. A heavy conidial suspension is prepared in glycerol (10% w/v) which is treated as in the case of yeasts.

Mycelial fungi are grown in media supplemented with 5% (w/v) glycerol (Fig. 1 E). Strains that do not tolerate the lower water activity caused by the cryoprotectant may be grown without glycerol and flooded with a 10 % (w/v) glycerol solution shortly before processing. A sterile straw open at both ends is now used to punch the mycelium with the agar from near the margin of the colony (Fig. 1 F). This procedure is repeated until the straw is filled completely (Fig. 1 G). The straw is either left open at both ends and transferred aseptically to a sterile cryotube or it may be sealed.

Freezing

To obtain a freezing rate that is close to the theoretical optimum of 1 - 10 C per minute, the cryotubes are either transferred to a mechanical deep freezer at -70 C for two hours in a styrofoam box of 2 cm wall thickness or placed in the gas phase of a liquid nitrogen tank for about 40 min.

Thawing

For revival, one straw at a time is removed from the frozen cryotube; the sealed straws are transferred into a 50ml glass beaker with warm water (30 C), open straws filled with mycelial fungi are thawed directly on agar slants at room temperature (22 to 25 C). Sealed straws may be surface sterilized by immersion into 70% ethanol (v/v), before they are opened with sharp, sterile scissors or pincers. The cell suspension is withdrawn with a fine Pasteur pipette. Incubation is done at appropriate temperatures until growth is visible.

Selected references for further reading

Elliott, T.J. 1976. Alternative ampoule for storing fungal cultures in liquid nitrogen. *Trans. Brit. Mycol. Soc.* 67, 545-546.

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