

CRYOPRESERVATION OF BACTERIA WITH SPECIAL REFERENCE TO ANAEROBES

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Introduction

The methods used in the maintenance of stock cultures of microorganisms usually involve serial subculturing or other simple methods of preservation which are not only time consuming but can also result in genetic instability. Cryopreservation of microorganisms in liquid nitrogen at -196°C is a very reliable method and is generally considered superior to other preservation methods. Bacteria preserved in liquid nitrogen normally show high survival rates and good strain stability during long-term storage. In liquid-nitrogen storage of microorganisms polypropylene cryotubes, glass vials, glass capillaries and polypropylene straws are generally used. In the method described here, screw-cap polypropylene cryotubes and mini-screw cap glass ampoules have been used. The latter are also suitable for the preservation of bacteria under anaerobic conditions. The method is simple, effective and economical with respect to the storage space and costs. In the preservation of anaerobes, generally no continuous stream of nitrogen gas, no anaerobic chamber or glove boxes are required. This method provides a suitable model for cryogenic storage of many fastidious and delicate bacteria.

General considerations for successful cryopreservation

Several factors can affect cell viability and stability during cryopreservation. During cryopreservation, dehydration of cells results and osmotic imbalance is created due to the changes in the concentration of salts and other metabolites. During the cooling process rupture of the cellular membranes can also occur by the formation of large ice crystals. Successful preservation can be achieved by the use of cryo-protective agents (such as dimethylsulfoxide, glycerol), maintaining a controlled rate of cooling (about 1°C per minute to about -30°C) and an appropriate rewarming protocol (rapid thawing in a 37°C water bath which takes about one minute for a glass ampoule and somewhat longer for a plastic vial). In practice, a relatively slow cooling rate can be easily obtained by keeping ampoules / vials in mechanical deep-freezers for 1-2 hours or in the neck of the liquid nitrogen storage unit for some minutes and then lowering containers into it. It is, however, not good practice to plunge

cultures directly into liquid nitrogen, as the liquid nitrogen may seep into any imperfectly closed or sealed capillaries, ampoules, or vials containing the bacterial suspensions. On removal from storage, nitrogen (inside an ampoule) will virtually instantly change to the gaseous phase causing an explosion. For safety reasons it is thus recommended that cultures should be stored in the gas phase of liquid nitrogen.

While preparing cells for cryopreservation several factors such as optimal growth conditions, physiological state of the cells (preferably from the late logarithmic to early stationary phase of growth), high cells density (10^6 to 10^8 cells per ml) should be considered as these can affect cell viability after cryopreservation. After mixing, cell suspensions should be kept for equilibration with the cryoprotective agent. For harvesting, liquid cultures are centrifuged. However, vigorous pipeting and high-speed centrifugation should be avoided and cells should be handled gently. Viability assays should be performed on all cultures before and after cryopreservation to assure long-term viability. To assure purity, identity of the preserved cultures should be verified and after freezing cultures should be recharacterized to assure their stability. Safety precautions should be observed when removing an ampoule from liquid nitrogen. The face shield, laboratory coat and insulated gloves should be worn as protection against liquid nitrogen splash and exploding ampoules. The level of the liquid nitrogen in the containers should be checked on a daily basis and maintained to a constant level, as any drop in liquid nitrogen level below a critical volume can result in damage due to the warming of the samples.

Materials and Methods

Equipment

Screw-capped plastic cryovials of about 2 ml capacity (available from Nunc, Gibco Europe Ltd., or Nalge Company). These are supplied in sterile packings. These are not suitable for repeated use. Screw-cap glass ampoules (10 x 30mm) of 2 ml capacity (available from Varian GmbH, Darmstadt, FRG). These are generally used for autosamplers in gas chromatography and are provided with rubber septa and plastic screw-caps with holes for the injection of the samples. It is recommended to use ampoules with black screw-caps and oxygen-impermeable butyl rubber septa. The ampoules are washed, rinsed with distilled water, tightly closed and autoclaved. Before use these are labelled with the numbers of the strains to be preserved. Liquid nitrogen storage tanks with cannisters, racks, canes, (supplied by Union Carbide, Air Liquide, France; Messer Griessheim, FRG; or else where) and precautionary arrangements like safety glasses, gloves etc.

Anaerobic facilities for the preparation of reduced media. Hungate tubes with septums (Bellco Glass Inc., 2047-16125). Butyl rubber overflow tubes (about 5mm diameter) with Luer Lock adapters at both ends and long syringe needles (10-15 cm in length). Sterile gas tight hypodermic Luer Lock syringes. Cryoprotective agent glycerol and dimethylsulfoxide (DMSO), reagent grade. Glycerol (20% w/v in H₂O) may be sterilized by autoclaving for 15 minutes and is stored in screw-cap bottles in dark. DMSO (20% v/v in H₂O) is sterilized by filtration (using a Teflon syringe filter) or can be autoclaved undiluted at 114 C for 10 minutes.

Preparation of cell suspension for freezing

The aerobic cultures to be frozen are grown in appropriate media (under well aerated conditions) and should be harvested in the late logarithmic to early stationary phase of growth. From agar slants the cultures are removed with a loop and gently suspended in sterile glycerole (10% w/v) or DMSO (5% v/v) to obtain a heavy cell suspension. Thick suspensions (10^8 - 10^{10} cells/ml) of liquid cultures are mixed in equal quantities with the double concentrated cryoprotective agents. For harvesting, the anaerobic cultures are centrifuged for 30 minutes at 4000xg in the screw-cap bottles in which cultures are grown. The supernatant is removed anaerobically under a stream of nitrogen gas using an overflow butyl rubber tube of about 5mm diameter with Luer Lock adapters at both ends and fitted with long syringe needles of 10-15 cm length (see Fig.1A). To obtain sterile nitrogen gas a sterile, cotton filled syringe is attached to a conduit connected to the N₂ gas (99.99%) cylinder. The pellet is resuspended carefully in ice cold sterile DMSO solution (5% v/v in H₂O). In the case of halophilic strains or cells which do not form a pellet a thick bacterial suspension (in growth medium) is mixed in the ratio 3:1 with ice cold sterile DMSO (20% v/v in H₂O). The cells are allowed to equilibrate with the cryoprotectant (15 minutes for DMSO, 30 minutes for glycerol) in an ice bath.

Filling of ampoules and freezing

While equilibrating, an aliquote of 1.0 to 1.5 ml of cell suspension is dispensed in to each plastic cryovial or glass ampoule. For anaerobes using a sterile gas-tight 5-10 ml syringe the ampoules are evacuated for anaerobiosis and to facilitate filling (Fig.1B). About 1 ml of thick cell suspension (equilibrated with the DMSO) is withdrawn with a 1 ml sterile oxygen free syringe (already flushed with nitrogen gas) and injected into each ampoule (Fig.1C). Immediately after the glass ampoules or cryotubes are clamped onto labeled aluminium canes, placed at -30°C for about one hour or for few minutes in the gas phase of liquid nitrogen. The canes are then placed in cannisters, racks or drawers and frozen by direct immersion in liquid nitrogen or in the gas phase of liquid nitrogen (Fig.1D).

Revival of cultures

The frozen ampoule is removed from liquid nitrogen. For partial thawing these are immediately immersed to the neck in the mini water bath at 37°C (Fig.1E) for a few seconds. After thawing the outer surface of the ampoules is dried by wiping and plastic vials are wiped with alcohol-soaked gauze prior to opening. For aerobic bacteria the screw-cap glass vials can be opened and flame sterilized at the neck. The thawed contents of the ampoule / vial are immediately transferred to fresh growth medium to dilute the cryoprotectant, which otherwise is lethal at higher temperatures. For anaerobes the septum of the glass ampoule is flame sterilized and with a 1 ml oxygen free syringe a small volume (about 0.05ml) of inoculum is withdrawn and injected into 5-10 ml liquid growth medium (Fig.1F). The rest of the cell suspension is immediately frozen again (a self made wax block rack, chilled to -30°C is used for transportation to the liquid nitrogen container see Fig.1G) in liquid nitrogen for later use. In this way one ampoule can be used for several repeated retrivals or inoculations. The DMSO which is often toxic during growth is diluted 100-200 times in the culture medium to a non

inhibitory concentration. The inoculated growth medium is incubated under appropriate growth conditions.

Estimation of viability counts

For aerobic bacteria, 0.5 ml of inocula is transferred to 4.5 ml of liquid growth medium and serial decimal dilutions are prepared. Plating and counting are done using standard methods. For the estimation of viable cell counts in anaerobic bacteria, 0.5 ml of inocula is transferred from the unfrozen (for cell counts before freezing) and from the thawed cell suspension (for cell counts after freezing) into prereduced 4.5 ml medium in screw-cap tubes (Hungate tubes with septa, Bellco Glass Inc., 2047-16125) and 6-8 serial decimal dilutions are prepared using oxygen free syringes and incubation is done under appropriate conditions. Agar roll tubes can be prepared for viable colony counts determination if such facilities are available. Colony counts on agar plates can be performed in an anaerobic glove box or anaerobic jars. Single plates can be incubated anaerobically in anaerobic Bio-bags (Type A, Marion Scientific Corporation, Kansas City, MI, USA). In the case of viable colony counts in agar roll tubes or on plates the number of colonies are counted from each dilution and average colony forming cells per sample are calculated.

For cultures which are difficult to grow in, or an agar, only liquid dilutions series are made. In this case the number of cells is determined using the most probable number method (MPN). For comparison the viable cell counts before freezing and after freezing are recorded and percentage survival is calculated.

Selected references for further reading

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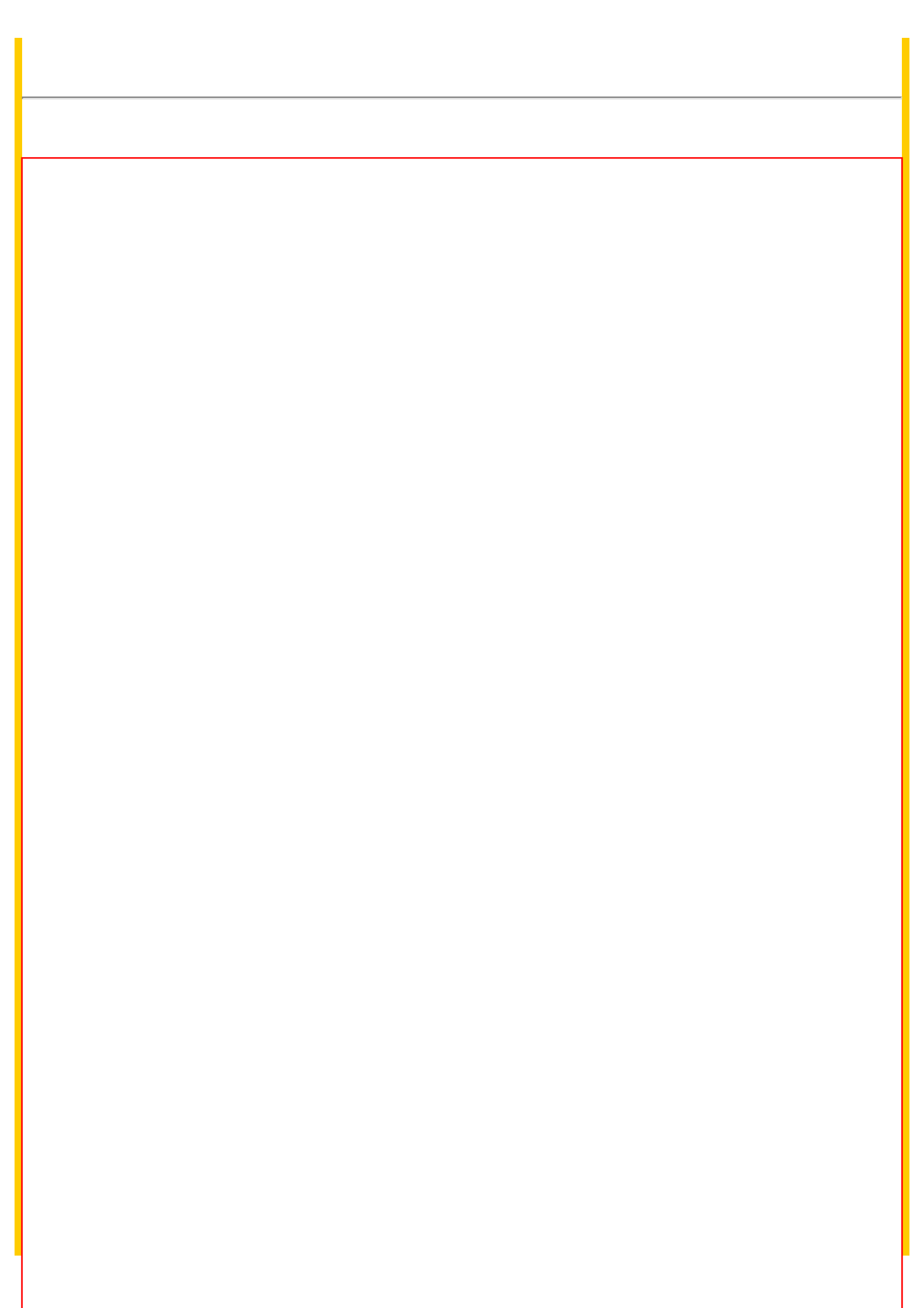
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