FREEZE-DRYING OF MICROORGANISM USING A SIMPLE APPARATUS

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Introduction

The advantages of freeze-drying are obvious. It is a convenient method for the preservation and long-term storage of a wide variety of microorganisms. However, special precautions are needed for the preservation of microorganisms sensitive to desiccation, light, oxygen, osmotic pressure, surface tension and other factors. During several years of experimentation I have developed various new methods and have optimised freeze-drying conditions to achieve successful lyophilization of a large collection of difficult and fragile microorganisms.

Normally specialized equipment is required to create the conditions conducive to the freeze-drying process. The costs of such specialized equipment required for freeze-drying can be substantial, and thus the process may appear to be expensive inspite of its many advantages. A freeze-drying process is described here using a simple apparatus.

This method is based on a new freeze-drying method which has recently been described (Malik, 1988). Some effective protective agents (Malik, 1976 & 1988) for example skim milk and meso-inositol or honey or glutamate or raffinose, are used to suspend cells to be freeze-dried inorder to protect these against known freezing and drying injuries.

Several anaerobic bacteria which are sensitive to aerobic freeze-drying, can successfully be preserved using activated charcoal (5% w/v) in the suspending media along with the above protective agents (Malik, 1990).

With this simple method various yeasts, sporulating fungi, and bacteria can successfully be preserved. Many delicate microorganisms such as nitrogen-fixing bacteria like Azospirillum, Azotobacteraceae, Rhizobium, Xanthobacters, Spirillaceae, Vibrios, and others like Alcaligenes, Ancylobacter, Flavobacterium, Pseudomonads and few Rhodospirillaceae resulted in a fairly good viability and stability after lyophilization and during storage. However, relatively low viability results in the case of various yeasts such as Lecosporidium, Sporobolomyces, Rhodosporidium, Zygosaccharomyces and fragile bacteria like Aquaspirillum, Spirosoma, Flectobacillus, and others, as compared to the lyophilization done under standard conditions (Malik, 1988 & 1990). The method is simple and the equipment described here can be easily constructed in most laboratories.

Material and Methods

Preparation of freeze-dried skim milk ampoules

Ampoules of neutral glass (45 x10 mm) are washed in a detergent, then rinsed in distilled water and are air dried. For double vial preparation the ampoules are labelled and filled with 0.5 ml of 20% (w/v) skim milk
(Bacto-Skim milk Difco 0032) containing 5% meso-inositol or 5% raffinose or 10% honey. The ampoules are loosely plugged with non-absorbent cotton wool and sterilized at 115°C for 13 minutes. These are frozen at about -30 to -40°C for a few hours and are freeze-dried for about 6 hours, as described under the freeze-drying procedure (primary freeze-drying). Large batches of ampoules should be avoided but this depends upon the capacity of the freeze-drying system (vacuum pump, condensing tempertaure of the cold trap, etc.).

Preparation of protective agents

Solutions of most effective protective agents like meso-inositol (5% w/v), honey (10% w/v), sodium glutamate (5% w/v), raffinose (5% w/v) are prepared in distilled water, filter sterilized and stored at 4°C. For use these are selected according to the storage temperature available for storage of freeze-dried ampoules. For more details see Malik, 1988.

Preparation of cell suspension for freeze-drying

The cultures are grown on appropriate media until the late logarithmic phase of growth. A thick cell suspension (at least 10⁸ cells per ml) is prepared in an appropriate protective medium. In the case of liquid cultures the cells are harvested by aseptic centrifugation for 30 minutes at 4000 xg in screw-cap bottles and the pellet is suspended in a protective medium to yield a heavy cell suspension. The ready cell suspensions are kept in an ice bath before filling the ampoules.

Filling of ampoules and slow freezing of cell suspension

The already freeze-dried skim milk ampoules are cooled to about -30°C for 1-2 hours. To each ampoule about 0.03 ml (1 drop with a Pasteur pipette) of ice cold cell suspension is added aseptically on to the skim milk plug with care so as not to touch the sides of the ampoules. The ampoules are quickly placed again for 1-2 hours in a deep-freeze and are frozen at approximately 1-2°C/min to about -30°C. This is easily achieved if ice cold samples are placed in commercialy available deep-freezers.

The freeze-drying procedure

Freeze-drying involves the removal of water from frozen cell suspension by sublimation under reduced pressure. The outline of the freeze-drying procedure and the major steps involved are shown in Fig. 1.

The cold trap tube (a U-shaped thick glass or preferably a metal tube of about 2-3 cm dia and about 30 cm length), is connected with the vacuum hose (preferably the U-tube is filled with blue/dry silica gel) and is placed in a metallic beaker as shown in Fig. 1 A. It is chilled to about -35°C. A cooling mixture of ethylene glycol: water (1:1) is placed for few hours in a deep-freezer and is cooled down to about -30°C (preferably to -40°C). This is poured into the cold trap to give a maximum depth. The cooling mixture is also sealed in deep-freeze plastic bags and is cooled down or frozen in a deep-freezer or over liquid-nitrogen. This super cooled or frozen coolant in the bags is added to the cold trap and the bags are changed periodically (preferably after every 20-25 min) throughout the lyophilization run in order to maintain the temperature at a minimum level. Commercially available anti-freeze liquids used in car radiators as coolants are also satisfactory as an alternate for the low-temperature bath and cold trap. A double jacketed straight tube (exterior about 6 x 30 cm and interior about 3 x 25cm with outlet and inlet tubes of about 2 x 5cm, as shown in Fig. 1A) can also be used as a cold trap.

If available, dry ice is used for freezing the ampoules, to cool down the cold bath and is added to the cold trap periodically throughout the experiment.

For the double vial preparation freeze-drying is done in two stages involving primary freeze-drying and secondary drying. When the condenser (cold trap) temperature has reached about -35°C the frozen (to about -30C) ampoules are transferred quickly to the chilled metallic evacuation jar, which is dipped to about 2cm depth in the cold bath containing coolant at about -35°C (Fig.1.A). The vacuum is switched on and the temperature of the cold bath is allowed to elivate. If available, a vacuum meter or controller can be attached between the vacuum pump and the evacuation jar to control the vacuum. Primary-drying is continued for
about 3 hours to achieve maximum desication (at about 1 to 0.1 torr or mbar). At the end, the vacuum is replaced with nitrogen gas (especially for ampoules which are not subjected to secondary drying and are not to be sealed under vacuum). At the end of the experiment the water collected in the cold trap is drained out or the silica gel is replaced.

Constriction of ampoules, secondary-drying and sealing

In a double vial system the ampoules (containing primary freeze-dried cell material) are sealed under vacuum in a soft glass tube. After primary freeze-drying of the ampoules the projecting ends of the cotton plugs are trimmed in level to the end of the ampoules and these are transferred into soft-glass large tubes (130 x 15 mm) containing blue silica gel and cotton plugs. For insulation small amount of glass wool is also pushed down along with the ampoule (inner vial) to the bottom of the large tube (outer vial). This outer tube is then constricted, on a low flame by hand or by using Edward Ampoule Constrictor, 20-25 mm above the glass wool to avoid burning of the cotton plug of the inner vial. The constricted tubes are then attached to the manifold and mounted on a metallic evacuation jar. This operation is illustrated in Fig. 1 B. The vacuum is switched on and secondary drying is conducted for 1-2 hours (at 0.1 to 0.001 torr). The pink silica gel in the outer tubes will turn again to blue at this stage. The constricted outer tubes are carefully sealed, by hand or by using a Flaminaire blow torch, one by one maintaining vacuum (Fig. 1 C). To avoid cracking of glass a flame containing oxygen should never be applied to such neutral glass that has not previously been warmed on a normal flame.

Revival of cultures from freeze-dried ampoules

During reactivation of preserved microorganisms it is recommended to use the most favourable media and growth conditions. During several years of experience, I have observed that in the case of sensitive microorganisms when the preserved (lyophilized or cryogenically stored) cultures are revived, the counts on agar media are usually lower than in liquid media and similarly agar media of higher surface tension (such as nutrient agar) usually results in lower viable counts than mineral media of relatively lower surface tension. The contents of the freeze-dried ampoule are thus dissolved in sterile liquid growth media and incubated at a relatively lower temperature than the optimum growth temperatures. A few cultures may exhibit a prolonged lag period and thus are incubated for relatively longer periods. Normal growth usually appears after a second transfer into fresh medium. In a few cases growth is inhibited by the high concentration of protective mixture used during lyophilization. During reactivation the presence of activated charcoal in the suspending media results in higher survival recoveries, and the reactivated cultures grown in the presence of activated charcoal prove much more stable and could be maintained relatively longer as living cultures (Malik, 1990).
The use of activated charcoal as an adsorbent of harmful radicals in suspending media for the reactivation of lyophilized anaerobes is also recommended.

Estimation of viability and stability

Survival recoveries are checked before freeze-drying, immediately after freeze-drying and after storage. For the estimation of viability counts serial dilutions are prepared in liquid media. From each serial dilution 0.1 ml volumes are plated on appropriate growth agar media plates. The number of colonies are counted from the plates and average colony forming units per sample are calculated. The revived cultures are also observed for mutation, change in colony morphology or other characters.

Long-term storage

Stability of freeze-dried cultures during storage is very important. A high level of residual moisture content or exposure to oxygen have detrimental effects on the freeze-dried product. Freeze-dried material is hygroscopic and its exposure to moisture during storage can destabilize the product. The higher the storage temperature, the faster a product will degrade. Thus, the storage of freeze-dried cultures at lower temperatures will extend their shelflife. The unsealed freeze-dried ampoules can safely be stored for several years at about -30C. It has been observed by the author that similar viability counts were obtained after 4-5 years of storage when unsealed freeze-dried culture were maintained at -30C as compared
to the freeze-dried cultures which were sealed under vacuum and were stored at 9C (Malik, 1976).

Selected references


