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# Conserving Microbial Diversity: Practices, Trends and Beyond

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Interests in preservation of microbes for *ex situ* conservation have grown hand in hand with the microbial diversity studies flourishing worldwide. For a successful exploration study, preservation of isolated strains without any change in original traits is as important as collection of pure culture of microbes from different habitats, their characterization and exploration for economical use. Preservation of microbes is also important to realize the potential of characterized strains on a large scale. This review focusses on the different methods available for preservation of microbial strains for short to long term. In addition, specific preservation techniques for certain microbes and problems and concerns in the routinely used preservation techniques and revival of preserved microbes have been dealt in detail. The review highlights the importance of research in the area of preservation of microbes.

**Key Words: Conservation, Cryopreservation, Lyophilization, Microbial Diversity, Mineral Oil Storage**

## Introduction

Microorganisms are vitally important life forms on the earth for humankind not only because of their ubiquitous nature and cosmopolitan presence but due to their obvious role and direct involvement in the natural biogeochemical cycles, cycling of biologically important elements, minerals and nutrients, food web, environmental remediation, crop production or protection. Therefore, acquisition, characterization, cataloguing and conservation of microbes from different habitats are most important task for any culture collection. The World Data Centre for Microorganisms (WDCM) currently lists 820 culture collections, in 78 countries, holding more than 3,348,121 microbial strains, registered with the World Data Centre for Microbes. At present, WDCM database of culture collections encompass 1,521,992 bacteria, 952,933 fungi, 32,839 viruses and some of them are cell line (42,223). The status of Collections of Asian countries and its holdings depicts that India ranks third after Japan and China with the total holdings of 221,241 accessions and is 5$^{th}$ in the top 20 ranking countries maintaining the microbial culture collections. In India, Microbial Type Culture Collection (MTCC), Chandigarh, National Centre for Microbial Resource (NCMR), Pune, National Agriculturally Important Microbial Culture Collection (NAIMCC), Mau, National Fungal Culture Collection of India (NFCCI), Pune, National Collection of Industrial Microorganisms (NCIM), Pune and Indian Type Culture Collection (ITCC), New Delhi are the important microbial resource centres catering the needs of the research and industrial applications. Recognizing the importance of industrial applications of microorganisms, three culture collections in India viz. MTCC, NCMR and NAIMCC have been recognized as International Depository Authority (IDA) for patent purposes under the Budapest Treaty by World Intellectual Property Organization (WIPO). The conservation of microorganisms is key to their application in agriculture and industry. Microbial culture collections in India and abroad generally follow both short- and long-term preservation methods which are subsequently supplied to a wide range of clienteles including academia and industry for diverse applications and basic research. In this paper, we aim to briefly describe various methods of *ex situ* microbial preservation and future directions to develop more efficient techniques to conserve more of the microbial diversity for future applications.

## *Ex-situ* Conservation of Microorganisms

The importance of microbes in modern civilization is incomparable due to their multifaceted application in food, pharmaceuticals, and biotechnology industry. Working with microbes requires isolating, characterizing, and preserving the potential ones for a longer period of time. Microbes have a brilliant capability of surviving

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long-term preservation with the least effect on their physiology than any other organisms (Hawksworth and Kirsop, 1988). This gives the opportunity to maintain microbial cultures for an almost indefinite time. The diversity of microorganisms is enormous and so are the methods of their conservation. Some of the microbes can survive in sterile water itself (e.g. *Ralstonia*) whereas some of the classes of microbes are very difficult to preserve for a long time (e.g. non-sporulating fungi and cyanobacteria). Different preservation methods for microorganisms are used, such as slant, stab, mineral oil stock, cryopreservation, lyophilization, drying in an inert material, etc. The following sections briefly describe various preservation methods for microbial cultures-

### Short-term Preservation

Researchers follow varied techniques for preserving microbial strains for a short period of time. It is the simplest method for preserving microbes, however, has more chances of developing mutants, loss of culture, and compromising culture purity. Different methods used for short-term preservation are as follows-

### Preservation in Slants

Both bacterial and fungal cultures growing on agar slants can be stored at 4-8$^{o}$C for a period of 6-8 months. Some additives such as acacia gum could further enhance survival. Oil overlay is also practiced to improve the survival of slants (Nakasone *et al*., 2004).

### Storage in Sterile Water

Many bacteria, as well as fungi, show stability in sterile water. It is inexpensive and successfully reported for preserving members of fungal groups like oomycetes, basidiomycetes, ectomycorrhiza, ascomycetes, yeast, etc. for varying periods. Though the survival varies from class to class, a maximum survival of upto 10 years is reported in sterile water for wood-inhabiting ascomycetes when stored at 20 ºC (Johnson and Martin, 1992). Bacteria such as *Ralstonia solanacearum, Pseudomonas fluorescens, Xanthomonas campestris,* and *Staphylococcus aureus* are also reported to have different degrees of survival in sterile water.

### Mineral Oil Storage

Fungal cultures are preserved submerged in high-quality mineral oil. The storage can be done at ambient temperatures. Cultures can be preserved for several years immersed in mineral oil. The high-density mineral oils restrict the oxygen diffusion and thereby slow the metabolic activities leading to survival for a longer period. This technique is suitable for nonsporulating fungi which can't be preserved through cryopreservation and lyophilization.

### Long-term Preservation

Continuous sub-culturing affects the cultural purity as well as compromise the important traits of the microbes, thus long-term preservation methods are followed to keep the microbial cultures for a period of 5-10 years. Preservation in -80$^{o}$C, -196$^{o}$C, and lyophilized storage are done for long-term storage. The long-term storage in form of cryopreservation and lyophilization is suitable for many microorganisms, the research has to be poured into developing protocols for preserving recalcitrant microbes.

### Lyophilized Storage

Lyophilization/freeze-drying is one of the most reliable methods for the long-term preservation of microbes. This is practiced regularly in most of the culture collections due to low maintenance and ease of transport. In the process of lyophilization, the cell suspension is frozen, dried under vacuum, and sealed. This dry powder under vacuum can be kept at ambient temperature for a period of 5-10 years, or even more, depending on the type of culture. Stability in lyophilized culture is achieved by the addition of cryoprotective agents. Skim milk (10%) is commonly used as a cryoprotective agent. Other cryoprotectants like inositol, sucrose, etc. are also used. Vacuum is maintained in the lyophilized culture vial throughout the preservation time. Though the lyophilized ampules can be stored at ambient temperatures, the viability can be further improved by storing the ampoules at 4 ºC in dark.

### Cryopreservation

Cryopreservation of microbes refers to conserving microbial cells at cryogenic temperature. Preservation at ultra-low temperature (-80°C or below) halts cellular activities and prevents damage to DNA and proteins. Halting the physiological activities of the cell helps in maintaining life for a longer period of time. Cryoprotectants such as glycerol (10-20%) and DMSO (5%) can prevent cellular damage due to the formation of ice crystals inside cells (Wowk, 2007). Polymer-based (polyethylene glycol) cryopreservation has also been reported for bacteriophages (Marton *et al*., 2021).

Different temperature storages are in practice viz. -80 and -196ºC. The storage at -196ºC is done through liquid-$N_2$ and requires constant refilling. As the rate of survival of the cryopreserved microorganisms decreases with the increase in temperature, preservation below -80°C is not recommended. The microorganisms can be preserved in 10-20% glycerol stocks for 8-10 months period (some microbes can survive even longer) at -20°C which, however, is not suitable for long-term preservation. Besides storage temperature, the success of cryopreservation depends largely on the rate of cooling, cell size, cell wall physiology, the permeability of water, and nature of the cryoprotectants used. To cover the preservation of diverse groups of microbes, specific attention is required to optimize suitable cryoprotectants and other conditions. The length of survival also depends on the rate of freezing and thawing.

## Specific Preservation Techniques and Modern Development in Preservation

Apart from the above-mentioned routine approaches, specific preservation techniques have also been developed for recalcitrant microbes which have low viability under common long-term storage protocols. To cover the preservation of diverse groups of microbes, specific attention is required to optimize suitable cryoprotectants and other conditions after considering the physiology of such microbes. The vegetative hyphae of saprophytic fungi *Lentinus crinitus* successfully cryopreserved using wheat grain along with other cryoprotectants (glycerol, DMSO, sucrose, glucose) (Bertéli *et al*., 2022). A collective effort has been taken by the European culture collections under the EMbaRC project for improving the survival and storage of lyo-recalcitrant bacteria (Peiren *et al*., 2015). This study concluded that horse serum supplemented with trehalose and skim milk supplemented with trehalose were better-suited lyoprotectant. Cryopreservation for microalgae is still a challenge. Recent reports suggest that cold acclimation at 4°C for 24 h before lyophilization along with controlled freezing, plunging, and thawing using an accurate programmable freezer could enhance the survival and storage of microalgae *Ettlia* sp. (Ha *et al*., 2019). In the case of anaerobic microorganisms, handling and storage is difficult. A novel kit-based strategy called BIOME-Preserve is used for transporting and preserving anaerobic microorganisms such as *Bifidobacterium*, *Bacteroides*, *Blautia*, and *Anaerobutyricum* (Hyde *et al*., 2022) at cryogenic temperatures. Resting structures of some fungi such as sclerotia or microsclerotia can be preserved for 2-3 years at 4°C. Soil fungi such as *Magnaporthe, Physarum, Cylindrocladium*, etc can be preserved by this method (Singleton *et al*., 1992). Preserving fungi on inorganic substrates such as grains, agar strips, wood chips, filter paper, straw, etc. is also a useful practice (Nakasone *et al*., 2004; Bertéli *et al*., 2022). Preservation in the sterile soil-sand mixture is also practiced for fungi like *Rhizoctonia solani* (Sneh *et al*., 1991). Silica gel can be effectively used for sporulating fungi.

## Problems and Prospects in Microbial Conservation

The methods used for both short- or long-term preservation of microorganisms suffer from certain disadvantages. Short-term preservation like subculturing on slants or maintenance of live cultures in liquid medium are prone to contamination and affect the stability of important traits due to mutation. The loss of traits after subculturing or maintenance as live cultures are more frequent in case of plasmid borne characters. However, these methods are the most popular ones as they do not require sophisticated instruments like ultra-low freezers or freeze dryers. The key to the preservation of fungi in mineral oil is the quality of the oil used. Ideally the oils should have a density 0.83-0.89 g/cm$^3$ which otherwise may not be effective in preservation. Many of the fungi preserved in mineral oil show slow revival and may also require a number of subcultures to remove the oils. However, there are also chances of contamination of the overlaying oil with airborne fungal spores if the tubes containing the fungal cultures are not kept tightly capped and not placed in a clean place. Mineral oil preservation is more effective when kept at low temperature.

Cryopreservation of microbes in glycerol or DMSO stock is yet another popular approach. Although many other cryoprotectants have been used but glycerol or DMSO have been mostly used. It has been reported that a number of aerobic bacteria like *Staphylococcus, Micrococcus, Pseudomonas, Streptococcus, Lactococcus, Corynebacterium* and *E. coli* show toxic effects with 10% DMSO (Fomin *et al*., 1973). Even some microalgae like *Chlorella* have shown toxic effect at very low concentration (<2.5%) of DMSO (Morris, 1976). Some microorganisms like *Methylomonas, Methylocystis* have been reported to show very minimal cryoprotective effect while a few others like *Rhodospirillum, Micrococcus,*

*Pseudomonas* have even shown toxicity to glycerol (Fomin *et al*., 1973; Green and Woodford, 1992). A large number of other cryoprotectants like polyalcohols, saccharides, heterocyclic compounds have also been used as cryoprotectants in microbial preservation. Moreover, a combination of cryoprotectants may be more useful for longer preservation as compared to using a single or routine one. Cryopreservation under temperature at or below -80°C requires ultralow temperature freezers or liquid nitrogen which may not be available at every microbiology laboratory. The rate of cooling and thawing is another important factor affecting the cell viability. Cell type and cell size is also crucial for cryopreservation as the response to low temperature varies with such factors. Initial cell density should be high enough as the microbial cells will die throughout the storage period.

Though lyophilization is very successful in preserving many bacteria, actinomycetes, archaea, and fungi, some of the microbial classes such as non-sporulating fungi, yeast (e.g. *Lipomyces, Leucosporidium, Brettanomyces*), and bacteria (e.g. *Clostridium botulinum, Helicobacter pylori*) can't survive lyophilization with routine protocols (Smith *et al*., 2008). In addition to cell density and type of cryoprotectants used during freeze drying, the growth phase of the cells is also a critical factor for survival of bacteria upon lyophilization. Desiccation tolerance of microorganisms is also important factor for freeze drying as desiccation sensitive cells may lose the viability in a short period. Rehydration of the freeze-dried microbial cultures is also crucial. Use of inappropriate rehydration media may also result in poor or no growth during revival. Usually, 10% non-fat skimmed milk can be a good rehydration medium.

In most of the laboratories and culture collections, a generic preservation methodology is followed which may, however, not be very useful always. The physiological responses of the microbial cell to low temperature, desiccation need to be considered along with the type and organization of the cell. As the known microbial diversity is only minor fraction of the actual diversity, we need to focus more on developing novel methodologies to preserve more diverse microflora. In nature, even the recalcitrant microbial cells can survive for a considerable period which otherwise either cannot be preserved in laboratories at all (e.g. obligate pathogens) or can be preserved for a very short period. Therefore, mimicking the natural conditions like preserving in freeze dried host or soil or sand or natural polysaccharides may help to preserve for longer period. Certainly, other approaches like optimizing conditions of cryopreservation or lyophilization and using appropriate cryoprotectants should also be carried out. Besides freeze drying, other drying technologies like fluid bed drying or spray drying should also be largely tested for wide range of microorganisms.

## Conclusion

The research on preservation of microbes is not given its due credit, even after understanding the importance of microbes in various fields. In most culture collections worldwide, a generic method is followed for medium term and long term preservation. This is not advisable especially in case of storage of cells in deep freezers (-20ºC and -80ºC) in the presence of cryoprotectants like glycerol and lyophilization. With regards to medium term storage in deep freezers methodologies are to be standardized for efficient preservation by optimizing the different cryoprotectants and their concentration for different groups of microorganisms. Similarly, for lyophilization, methodologies are to be optimized with different lyoprotectants for different group of

### FUTURE DIRECTIONS

- Instead of hit and trial with methods or cryoprotectants, a biology/physiology driven approach should be taken up. Based on the biology of the microbes and environmental tolerance, the method of preservation should be chosen
- The natural survival of the microbes in diverse environments should be mimicked for longer preservation.
- Efforts should also be devoted towards optimizing conditions and media to induce resting structures which can be easily preserved for longer durations
- Developing long term preservation methods for obligate pathogens retaining their pathogenicity. Host factors should also be considered while developing such methods

microorganisms. Culture collections worldwide should in future focus on optimizing the routine preservation techniques of microorganisms of paramount importance. They should also workout on the methodologies for long term preservation of recalcitrant microbes like archaea and others.

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