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Review Article

Preservation of Viable Microorganisms in the Laboratory: An Overview of Basics, Methods and Practical Recommendations for Beginners

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Abstract

Preservation of viable microbial cultures in laboratory practice is necessary for performing both short-term and long-term projects with microorganisms *in vitro*. There is no universal method to preserve cultures of live microbial cells of different taxonomic groups. This brief review summarizes the basics of methods for short-term and long-term preservation of viable cultures of microorganisms *in vitro*. Practical recommendations are presented for the preservation of viable cultures of microorganisms, which can be useful in conventional microbiological laboratories.

Keywords: Microbial cultures; Low-temperature preservation; Subculturing; Viability

Introduction

The study of viable microorganisms in the laboratory begins with obtaining a culture of a certain strain. Such a culture can be obtained from an authoritative Culture Collection (Biological Resource Center), can be own isolate or the result of genetic modification of a strain obtained from an authoritative source. In any case, the ability to keep microbial cultures in a viable state, at least for the duration of a certain project, is a skill that is necessary for a fruitful work. However, *there is no universal method to preserve live microbial cells of different taxonomic groups in vitro*. This is due, firstly, to the specific features of various microorganisms, secondly, to different requirements for different storage duration and, thirdly, to the technical capabilities of laboratories.

Modern approaches for the preservation of microbial cultures in a viable state have hundreds of techniques. They have been described in numerous original papers, as well as in reviews and manuals devoted mainly to the work of Culture Collections [10,17,22,26,32,34]. Development of these techniques was the result of fundamental research in cryobiology [21,25], including in relation to microorganisms [7], as well as in the studies of the phenomenon of cryptobiosis (anabiosis, suspended animation) in different groups of living organisms [8,9,15,23]. Particular attention was paid to methods of long-term preservation of viable microorganisms for biotechnological production of probiotics [6,16] and microbial starter cultures (inocula) [2,14,29].

Diving into the "information ocean" of literature devoted to various aspects of this problem can be a fascinating and useful activity. However, for most people working with cultures of microorganisms *in vitro*, especially for beginners, the practical aspect of the matter is more relevant; namely, how to keep microbial cultures in a viable state in every particular case? To answer this question, it is helpful to have a general understanding of the basics that underlie the methods of preserving microorganisms in a viable state *in vitro*. This will make it possible to consciously choose or even develop the necessary technique in each specific case.

This review has three objectives. Firstly, to focus the attention of people who are just starting to work with microbial cells that their preservation in a viable state *in vitro* is an *issue that requires special attention*. Secondly, to briefly introduce *the basics* of methods of short-term and long-term preservation of viable cultures of microorganisms *in vitro*. Thirdly, to give practical recommendations on the preservation of viable cultures of microorganisms, which can be useful in most laboratories where there are basic conditions for working with microorganisms, as well as available equipment.

Basics of Preservation of Viable Cultures of Microorganisms

All methods of preserving viable cultures of microorganisms in laboratory practice are based on a *decrease in metabolic activity or even a complete cessation of all 'chemical reactions in cells*. This can be done primarily *by lowering the temperature and/or activity of the water.*

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However, it should be kept in mind that a living cell, firstly, is a system of finely balanced oppositely directed "main streams of life", anabolism and catabolism. Secondly, all cell structures are supramolecular complexes of proteins, lipids, nucleic acids and polysaccharides, which are organized due to relatively weak, non-covalent physical bonds (ionic, hydrogen, hydrophobic, and Van der Waals bonds). Both the rates of metabolic reactions and the state of supramolecular complexes strictly depend on the temperature and activity of water. It follows from this that significant changes in temperature and/or water activity relative to some "physiologically optimal" values can potentially cause structural and functional shifts in cells that will affect their viability or may cause mutations [7,11,25]. Thus, it can be concluded that when microbes are preserved in the laboratory using techniques that lead to a decrease in temperature and/or water activity, it is potentially possible that at least some cells may be damaged or even died.

The cells of microorganisms of different taxa and even strains have different susceptibility to damaging factors associated with a decrease in temperature and/or water activity. In addition, this susceptibility depends on the growth conditions of the culture, primarily on the cultivation medium and the growth phase.

There is a widespread opinion that one viable cell is enough to preserve a microbial culture. *In general, this is not true*. The microbial population (culture, sample) is heterogeneous, i.e., it contains (potentially) along with dominant cells representing the main features of the culture, a number of minor subpopulations with cells that differ from the dominant at the phenotypic and genotypic levels [1]. The preservation of culture under certain conditions may turn out to be a kind of selection, which can provide more favorable conditions for the prevailed survival of minor subpopulations.

Thus, the choice or even the development of a specific technique for the preservation of cultures should be aimed at finding optimal conditions of the procedures. Optimal here means, firstly, the maximum possible level of viability rate of the preserved population (culture, sample), which is defined as the relative proportion of viable cells, and, secondly, minimum "genetic drift", that is, a low mutation rate.

The Main Methods

Conditionally, three types of methods for preserving viable microorganisms in laboratory practice can be distinguished [31], namely, *subculturing*, *low-temperature preservation* (*cryopreservation*) and *drying*.

All methods can be implemented using a variety of techniques. Each of these techniques has some advantages in applying to a particular culture and/or storage conditions.

Subculturing is the most common and easiest way to maintain viable cultures in the laboratory. Normally, it is the action of transferring some or all cells from a previous culture to fresh growth medium. To increase the interval between passages to fresh medium, the cells are transferred to a hypometabolic state (hypobiosis). This state is created by a decrease in the content of nutrients in the medium or the availability of a gas phase in cultures grown on agar media under a layer of vaseline oil, and transfer to subzero temperatures (0 ± 4°C) [12,17,18,19,30,31].

A household refrigerator and conditions for sterile work are all that is necessary for the implementation of this method. The protocol for subculturing depends on the properties of the microorganism species. The duration of maintaining cultures by this method is an individual property of the strain. Usually, cultures are stored in this way for *several months*. For a longer preservation of cultures (up to several years), periodic passaging is carried out. The negative features of the method are the *labor intensity* and *the risk of contamination* of cultures during repeated passages.

Low-temperature preservation (***cryopreservation***) is a method of long-term storage of viable microorganisms. According to theoretical considerations, at temperatures below –70°C, which can be provided by modern cryogenic technology, two generalized processes occur in cells. Firstly, the water turns into a solid state, depending on the cooling rates, either into crystalline ice, or into an amorphous glassy state [21]. Thus, the water activity drops to zero. Secondly, the movement of all molecules slows down so much that almost all chemical reactions become impossible. Thus, at these temperatures, there is a theoretical possibility of infinite preservation of cells in an unchanged state.

It should be noted that at temperatures above –70°C, which are provided, for example, by the freezer of a household refrigerator (–10°C ÷ –20°C), it is possible that samples may not completely freeze with the formation of a eutectic mixture of ice and a solution of concentrated solutes. Under these conditions, the chemical transformations, including unfavorable ones for cells, cannot be excluded [24,25]. In addition, modern household refrigerators are equipped with an automatic periodic defrosting system of the freezer, which should be accompanied by at least partial defrosting of samples with potential unfavorable outcome for viability of cells.

The main problem of low-temperature preservation of living cells is that during cooling (freezing) and subsequent worming (thawing), damage to cells is possible [24,25]. Therefore, the methods of low-temperature preservation should include procedures that reduce the damaging effect of freezing-thawing during cryopreservation. These procedures include the use of cryoprotectants, the selection of optimal rates of cooling and warming (freezing and thawing) and immobilization of cells on some substrates (carriers), which can be, for instance, glass and ceramic balls [5,14,17,18,24,25,28,30,33].

Drying reduces water activity in a sample simply by removing it. This approach makes it possible to preserve viable microbial cultures in the most convenient form for storage and transportation. There are two main varieties of this method, drying from the frozen state (lyophilization, freeze-drying) and drying from the liquid state.

Lyophilization is one of the main methods of maintaining viable microbial strains in authoritative Culture Collections. The advantage of this method is a relatively high level of viability of cultures after lyophilization and the preservation of this indicator for a long time (several years). Lyophilization is carried out using special equipment. The samples are frozen, dried from the frozen state (ice sublimation at low pressure) and stored in sealed ampoules at low partial pressure of oxygen [3,4,17,22,27,28,30,33]. For lyophilization, relatively expensive equipment, special techniques and qualified personnel are requires. Therefore, this method cannot be recommended for widespread use in the practice of a conventional microbiological laboratory.

Drying from *a liquid state* of some types of microbial cells with preservation of their viability has been reported [19,22,34]. This also requires special techniques and equipment.

5. Practical Recommendations

Subculturing and low-temperature storage techniques can be implemented in a conventional microbiological laboratory.

Where to Begin

1. The care of the method of preserving the culture in a viable state must be taken before obtaining it.
2. It is advisable to request a source of culture provision and/or to search for literature on the methods of preservation of the particular culture in the laboratory.
3. In the case of the intended long-term storage of the culture (more than a year), it is advisable to use several methods of preservation/storage and at least 5 samples for each method. In the practice of microbial cultures collections, each culture (strain) is preserved using 2 – 3 methods.

The Preservation of Cultures in the Laboratory Should be Accompanied by Careful Documentation

1. It is necessary to label all samples clearly and reliably, the minimum entry on each sample is the strain number and date.
2. It is necessary to keep detailed documentation indicating the following minimum information: strain name, number of samples, storage address (room, refrigerator, number of cassettes, box, position in the box or in the cassette) date of storage, preservation procedure, dates and results of viability (or any other) tests, researcher name, any comments (in the case of computer documentation, save a copy of the documents on portable media).

The Following Recommendations can be of Use for Low-Temperature Preservation

1. Only freshly grown culture should be used for preservation.
2. Samples for preservation should contain the maximum concentration of cells (for suspensions it is about 10^7 – 10^8 cells/mL).
3. The most resistant to preservation and storage are cultures grown in a liquid medium to a stationary growth phase, and grown on a solid medium to well-expressed growth and/or sporulation.
4. The use of cryoprotectants.

The main cryoprotectants for the preservation of microorganisms are glycerin, dimethyl sulfoxide, sucrose, glucose and skim milk (many others can be found in Kirsop and Doyl 1991; Uzunova-Doneva and Donev 2004).

The final concentration of the cryoprotectant in the medium should be 10 – 15 %.

After mixing the cells with the cryoprotectant, it takes about 10 –20 min to balance the system, but no more (!) due to the possible toxic effect of the cryoprotectant.

When selecting cooling-warming (freezing-thawing) rates, the following practical procedures based on empirical observations can be used.

Cultures of prokaryotic microorganisms best tolerate “fast” freezing (approximately 200 – 300°C /min). This can be done by immersing a 0.2 – 0.3 mL sample in a special cryovial (for example, Nunc CryoTubes™) into ethyl alcohol cooled to –70°C in a low-temperature refrigerator (freezer) or into liquid nitrogen.

Cultures of eukaryotic microorganisms (for example, yeast) should be cooled “slowly” (approximately 0.1 – 10°C/min). At this rate, a 0.2 – 0.3 mL sample in the cryovial freezes in the air of the freezer with a temperature of –70°C. After freezing, such a sample can be stored in the same refrigerator or transferred to liquid nitrogen.

The warming (thawing) rate should always be maximum. In practice, a water bath with a temperature of 37°C is used for thawing, into which the cryovial with the frozen sample is immersed for 1 – 2 min.

After thawing, the sample should be transferred to the reactivation medium (growth medium) as soon as possible, in order to avoid the potential toxic effect of the cryoprotectant.

The effectiveness of the selected freezing-thawing procedure should be assessed by the level of viability in at least one sample next day. The control should be a sample of the same culture without freezing-thawing.

Finally, Some General Recommendations

1. Low temperature freezing is useful for storing samples as primary inoculums. This allows standardization of inoculation in terms of concentration and culture quality of microorganisms. For this purpose, several samples should be frozen according to the number of planned inoculations.
2. Cultures can be transferred for guaranteed long-term storage (deposit) to authoritative Cultures Collection (this procedure is paid for). This should be done, in particular, with cultures that, for some reason, may be of interest in future research. Deposition in an authoritative Cultures Collection is mandatory when patenting strains with useful properties or (bio)technology based on such strains.
3. If it is necessary to keep the cultures in a dried state, for example, for transportation, it is advisable to contact the authoritative Culture Collection, whose services include such work (this procedure is paid for).
4. On all issues of preservation of cultures of microorganisms, it is advisable to consult with specialists at the authoritative Culture Collection.

Lists of authoritative Culture Collections (Biological Resource Centers) can be found on the websites of The World Federation for Culture Collections (WFCC) (https://wfcc.info/home_view) and The European Culture Collections' Organization (ECCO) (https://www.eccosite.org/).

Conclusions

1. To work with microorganisms in the laboratory, knowledge of basics and methods for preserving microbial cells in a viable state *in vitro* is necessary.

2. The preservation of viable microorganisms in the laboratory is based on a decrease in metabolic activity or even a complete cessation of all chemical reactions in cells mainly by a decrease in temperature and/or water activity.

3. A certain strain of microorganisms should be stored in the form of a culture (representative population) containing a large number of cells.

4. Short-term (less than a year) preservation of microbial cultures can be carried out on/in a nutrient medium in a refrigerator at a temperature of 0 ± 4°C.

5. Long-term (several years) preservation of microbial cultures can be ensured by freezing to –70°C and below in a low-temperature refrigerator (freezer) or in liquid nitrogen using a special technique with certain cryoprotectants and freezing-thawing rates.

6. If it is necessary to store microbial cultures in a dried state, it is advisable to contact the service of an authoritative Culture Collection.

7. For guaranteed long-term preservation of microbial cultures in a viable state, they can be deposited in an authoritative Culture Collection.

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Conflict of Interest

The author declares that there is no conflict of interests.

Compliance with Ethical Standards

The article does not contain data obtained in the course of animal and human studies.

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