Methods of Cryopreservation in Fungi

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Abstract

Traditional method of the routine subculturing by transfer of fungal cultures from staled to fresh media is not a very practical means of storing large numbers of fungal cultures. It is time-consuming, prone to contamination, and does not prevent genetic and physiological changes. At present, besides freeze-drying (lyophilization), cryopreservation seems to be the best preservation technique available for fungi.

Keywords

Cryopreservation • Fungi collections • Liquid nitrogen • Perlite • Subculturing • Fungi storage

Introduction

Serious mycological (and generally biological) work requires a reliable source of cultures (i.e., well-defined and taxonomically determined starting material), which is ensured by its safe long-term storage. This implies the fundamental and growing importance of culture collections not only for preservation of the endangered genofond (and consequently the biodiversity), but also as a principal source of material for biotechnological processes, research, and teaching. The first and most important problem to be solved is the long-term maintenance of this material.

Collections of fungi were originally kept by serial transfers from staled to fresh media. This routine subculturing is not a very practical method for storing large numbers of fungal cultures. It is time-consuming, prone to contamination, and does not prevent genetic and physiological changes (degeneration, aging) during long-term and frequent subculturing [1]. Over the years, various storage methods have been developed in order to eliminate these disadvantages. Their common feature is at least partial suppression of growth and metabolism of the cultures. Among them, keeping fungal cultures in sterile water [2–8] was surprisingly efficient (especially with lower fungi, but also with some basidiomycetes) and experiences its revival. In some fungi, preservation under a layer of mineral oil, in silica gel, soil, or sand [9–13] was successful. These

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methods enabled a reduction in fungal growth and extended the time intervals between transfers to fresh media. Nevertheless, the method of serial subculturing is still used in collections with limited financial support or in the majority of other collections as a backup preservation method.

Searching for improved or new methods resulted through different intermediate steps in the introduction of lyophilization and cryopreservation of fungal cultures [14]. Application of the most extended method of culture preservation—freeze-drying (lyophilization), tested for sporulating fungi as early as 1945 [15]—is rather limited in the case of basidiomycetes and other fungi nonsporulating in vitro [16, 17]. Most attempts to revitalize dehydrated hyphae of fungi have failed, except for some successes [18–20]; nevertheless, the real absence of spores must be always carefully checked. Despite this, several attempts have recently been made using modified protocols [21–23] and the growing interest in this technique can be seen at present. An important role played in the whole process, besides the freeze-drying, is also the freezing rate and the lyoprotectant used [24, 25]. Freeze-drying of fungi has several important advantages over all other maintenance methods. Cultures can be stored easily in dense packing without any special requirements and need not be revived on agar slants prior to dispatch. The product is light, inactive, and dry, enabling easy distribution by mail.

The new or modified methods have been frequently used and evaluated [5, 6, 10, 26–31], but they are not generally applicable. Often a specific preservation protocol is necessary even for individual strains of the same species. At present, besides freeze-drying (lyophilization), cryopreservation seems to be the best preservation technique available for filamentous fungi [14, 26, 32]. A very comprehensive and detailed overview of the methods and results of the cryopreservation in microorganisms was published by Hubálek [33].

Cryogenic technique for long-term storage of large numbers of fungal species was introduced to ATCC in 1960 and the results have been very satisfactory [34, 35]. The technique was consecutively introduced to many other prominent collections, e.g., CAB International Mycological Institute [1], etc. In certain collections—e.g., IFO (Institute for Fermentation Osaka)—nonsporulating cultures of basidiomycetes are stored by cryopreservation at −80°C in electric freezers [36].

Mycelium and/or spore suspensions with or without a cryoprotectant in sealed glass ampoules were originally used for cryopreservation of filamentous fungi. Later, glass ampoules were replaced with safer polypropylene cryovials and/or straws. Agar blocks immersed in an appropriate cryoprotectant were originally used as carriers of fungal mycelium for the cryopreservation process [37]. A useful straw technique with agar miniblocks for the preservation of fungi in liquid nitrogen was developed by Elliott in 1976 [38] and improved by Stalpers et al. [39]. Another technique using straws in cryotubes without a cryoprotectant solution was described by Hoffmann [40]. A modified Hoffmann's technique was compared with the original agar block one in our paper [29]. Commercial preservation systems with polystyrene beads as carriers were used for cyopreservation of conidia of enthomopathogenic fungi [41] and of sporulating *Aspergillus fumigatus* cultures at −80°C [42]. Porous ceramic beads were employed for cryopreservation of several sporulating fungal cultures and for a *Saccharomyces cerevisiae* culture at −70°C by Palágyi et al. [43]. It is symptomatic that these techniques have not been used for nonsporulating filamentous fungi. In this context it should be mentioned that as early as 1978 Feltham et al. described a method of preservation of bacteria on glass beads at −76°C [44]. Some reports [45–47] indicate that cryopreservation at −80°C is suitable for many fungal cultures, including basidiomycetes. Nevertheless, Leeson et al. [48] state that to completely stabilize frozen cultures, the temperature must be sufficiently reduced to both minimize metabolism and prevent ice crystal formation, which can cause physical damage during storage. The temperature limit securing prevention of formation of such ice crystals is −139°C. This is why at present many culture collections start to keep their cultures at −150°C

in ultralow-temperature electric freezers, which are sometimes equipped with liquid nitrogen supply.

The cryopreservation process includes freezing and thawing and the protocol of these procedures plays an important role [49, 50]. In principle, there are two kinds of freezing protocols: a slow (controlled) one and a fast (uncontrolled) one, which both have been used for cryopreservation of fungi [51, 52]. Generally, too low freezing rates cause excessive dehydration and concentration of the solution leading to cell damage; on the contrary, too fast freezing leads to insufficient dehydration and formation of abundant ice crystals with lethal consequences. Nevertheless, different fungal cultures exhibit different sensitivities to freezing conditions and to the presence and concentration of cryoprotectants. A freezing rate of 1°C per minute is usually used for cryopreservation of fungi; in the author's experiments with sensitive mutant strains of *Agaricus bisporus*, the freezing rate 0.5°C per minute was successfully used.[1] Lately, cryomicroscopic methods have been used to study the process of freezing and thawing of fungal cultures [14, 24, 53]. Successful cryopreservation depends on the cryoprotectant used [54]. At present, dimethylsulfoxid and glycerol are the most widespread [55]. The method of cryogenic culture maintenance seems to be mostly successful also in nonsporulating cultures [14, 32, 56, 57]. An overview study on the influence of the cryopreservation process on survival of taxonomically very broad spectrum of fungi published by Smith [14] showed that there was no obvious link between taxonomic grouping and the response of the fungi to freezing and thawing. This was confirmed in our study [31]. Similar studies were carried out also in edible mushrooms *Lentinus edodes* [58] and the genus *Pleurotus* [59]. Davell and Flygh [60] showed that even an ectomycorrhizal fungus *Cantharellus cibarius* can be successfully cryopreserved when a sufficient number of cryoprotocols is tested. Cryopreservation of spores of vesicular–arbuscular mycorrhizal fungi was described by Douds and Schenck [61].

Beyond survival, another principal requirement for the successful preservation of fungal strains is maintenance of their genetic and physiological features, such as growth, morphology, and metabolite production. In our experiments with some white-rot basidiomycetes, no negative effect of cryopreservation or the used cryoprotective on production of ligninolytic enzymes was found [62]. The complete revival of cryopreserved cultures (evaluated mostly by measuring the colony diameter) is generally still uncertain. The survival rate varies between 60 and 100% [21, 58, 59, 63, 64]. Only a few studies of the genetic stability of cryopreserved fungi have been performed. Singh et al. [47] confirmed the genetic stability of 11 cryogenically preserved edible mushroom strains by comparing random amplified polymorphic DNA (RAPD) and internal transcribed spacer (ITS) profiles. Using polymerase chain reaction (PCR) fingerprinting, Ryan et al. [65] checked the genetic stability of several isolates of *Fusarium oxysporum* and *Metarhizium anisopliae*. Other studies include confirmation of the genetic stability of *Uncinula necator* conidia after storage at −80°C [45] and investigation of the influence of mid-term cryopreservation at −80°C on 15 isolates of 10 basidiomycete species, for which the DNA fingerprint patterns were unchanged [66]. All of these reports were solely based on fingerprinting methods, which are not suitable for the detection of minute yet important changes in the genome, such as point or short indel mutations. Rather, sequencing approaches are required to successfully detect these mutations. This approach was used in our recent study [67]. Considering the above data, there is a continuous need for developing, improving, optimizing, and combining of preservation procedures, because the present methods are not applicable to all fungal cultures. Although many of these fungi can be grown in pure cultures on solid media, their growth is often attenuated and their morphology and other characteristics changed, which can result in their complete loss. The number of characteristics evaluating the success of preservation should be increased.

[1] Homolka, unpublished results.

As mentioned previously, cryopreservation, namely in liquid nitrogen, seems to be the most reliable, safe, and prospective method of a long-term maintenance of most fungal species, especially those not amenable to freeze-drying. It is probably the only storage technique that can ensure genomic and phenotypic stability. But not even the aforementioned cryopreservation method is applicable to preservation of all fungal cultures in the present form. According to the literature as well as the author's personal experience, especially the maintenance of basidiomycetes is challenging. Many of these fungi do not form asexual spores, their dominant life form, the vegetative mycelium, is sensitive to environmental conditions and therefore not amenable to freeze-drying.

To address these issues, a method of cryopreservation using perlite as a carrier for fungal mycelia was developed in the author's laboratory (perlite protocol or PP) [28] and then successfully verified for 442 basidiomycete strains [30]. Perlite is a unique aluminosilicate volcanic mineral that retains substantial amounts of water that can be released when needed—a feature that seems to have a dominant effect on cryopreservation success. The PP can be used for cryopreservation of taxonomically different groups of fungi, including yeasts [31], and works relatively well for fungi that cannot survive other routine preservation procedures. Expanded perlite was used as a solid support in solid-state fermentations [68]; otherwise it is used in many applications, particularly in the construction, horticulture, and other various industrial fields. It is recommended as an efficient purifying agent and as a carrier for pesticides, feed concentrates, herbicides, and other similar applications.

Perlite Protocol (PP)

The protocol is suitable for maintaining a broad spectrum of fungal cultures of different origin. It was verified in several culture collections (e.g., in Finland, the Netherlands, USA, Czech Republic, etc.) with great success and now it is routinely used there.

Materials

1. Distilled water.
2. Agar Difco.
3. Glycerol p.a. Sigma.
4. Isopropyl alcohol 100% Sigma (alternatively).
5. Agricultural-grade perlite (Agroperlit, GrowMarket s.r.o., Prague, Czech Republic, http://www.growmarket.cz/produkt/agroperlit-8l)—particles 1–2 mm.
6. Dried wort extract Sladovit, Malthouse Bruntál, Bruntál, Czech Republic, diluted to a density of 4° Balling scale with distilled water (further wort in the text); or preferably MYA medium: Malt-extract Difco 25 g, Yeast-extract Difco 2 g, Glycerol p.a., Serva 50 g, distilled water ad 1,000 mL. pH adjusted to 6.5 with 1 M KOH solution.
7. Ethanol (70%).
8. Liquid nitrogen (LN).
9. Nunc CryoTube Vials 1.8 mL screw-capped (Nalgene/Nunc, Rochester, USA).
10. Cork borer.
11. Lancet and small spoon.
12. Rule for measuring of colony diameter.
13. Water bath.
14. Balling hydrometer (saccharometer) (alternatively).
15. Hot-air sterilizer.
16. Autoclave.
17. Refrigerator (about 4°C).
18. Deep-freezer (−80°C).
19. Microscope equipped with phase contrast.
20. Laminar flow box.
21. Thermostat (incubator) for 24°C.
22. Container for storing samples in liquid nitrogen (e.g., HARSCO TW-5K container, Harsco, Camp Hill, USA).
23. Programmable freezer for controlled freezing of cryovials with mycelia (e.g., IceCube 1800 freezer, SY-LAB Geraete GmbH., Neupurkersdorf, Austria); or alternatively Cryo 1°C Freezing Container "Mr. Frosty" (Nalgene Labware)[2].

[2] All chemicals and devices named can be replaced with other ones produced by other renowned companies.

Methods

The methods given below describe general procedures for cryopreservation of fungi using the perlite protocol (PP).

Strains (Cultures)

The starting cultures are kept on wort agar slants or dishes (wort 4° Balling, 1.5% agar Difco) at 4°C or other media suitable for the growth of the strains destined for the procedure (e.g., MYA medium with 1.5% agar, etc.) and transferred to the fresh medium every 6 months.

1. Prepare an agar medium, sterilize it in an autoclave (121°C, 20 min.), pour it into sterile plastic Petri dishes (diameter 100 mm, 30 mL per dish), and let it cool down in a laminar flow box.
2. In a laminar flow box cut out an agar plug (6 mm diameter) from the actively growing part of a colony on a Petri dish with a cork borer, place it on a Petri dish with fresh medium using a sterile lancet and then let the dish incubate for 14 days at 24°C. Then put the dish(es) into a refrigerator and keep it at about 4°C.

Culture Preparation and Freezing–Thawing Protocols

Fungal cultures are grown directly in firmly closed sterile plastic cryovials (1.8 mL) with 200 mg of perlite (Agroperlit, agricultural grade) moistened with 1 mL of wort (4° Balling) or other medium (e.g., MYA) enriched with 5% glycerol as a cryoprotectant. For sterilization of the cork borer, lancet, and small spoon use a hot-air sterilizer (150°C, 30 min.).

1. Distribute perlite into cryovials (200 mg per vial), flood it with 1.8 mL of the medium enriched with 5% of glycerol, and sterilize vials in autoclave (121°C, 20 min.). In a laminar flow box cut out an agar plug (6 mm diameter) from the actively growing part of a colony on a Petri dish using a cork borer, place it using a sterile lancet on the surface of perlite in the cryovial, close the vial firmly and let it incubate for 14 days at 24°C.
2. Freeze the cryovials with perlite overgrown by mycelium in a programmable freezer (or alternatively in a "Mr. Frosty" container in a deep-freezer) to −70°C at a freezing rate of 1°C per minute. Then place them in LN in a container.
3. Take the stored frozen cultures in cryovials out of the LN container, transfer them to a water bath (37°C), and leave them there until the ice is completely thawed (thawing—reactivation of cultures). Prior to opening, disinfect the surface of cryovials with 70% ethanol.

Viability Test

1. After thawing, separate at least partially the perlite particles overgrown with mycelium by shaking, the content of the cryovials (two parallels of each strain) divide into three approximately equal aliquots each and these plate onto wort (or an other) agar medium in Petri dishes (diameter 100 mm) using a small sterile spoon.
2. Incubate the cultures in Petri dishes at 24°C for 14 days. Strains exhibiting survival of at least four out of six separate aliquots are considered viable.

Growth Estimation and Morphological Analysis

1. Growth of cultures measure as a mean diameter increase of a growth-covered zone (in mm) during a 14-day incubation at 24°C on the respective agar medium in Petri dishes (diameter 100 mm) inoculated with perlite aliquots from cryovials before freezing and after reactivation. Measure six zones (three aliquots from two cryovials) for each strain. The first occurrence of growth varies between frozen cultures, with some strains showing signs of re-growth within 2 days but most strains reactivating within 7 days after plating.

2. Use the same procedure except for freezing and thawing for growth measurement of the control.
3. Carry out the morphological analysis on control cultures and on those arising from the viability tests. Check the selected macroscopic features (colony color, reverse color, texture of the mycelium) and microscopic features (hyphal branching, presence/absence of clamp connections, presence/absence of hyphal vacuolization, etc.) using a microscope.
4. If possible, estimate also other characteristics of the resulting cultures (e.g., enzyme or metabolite production, etc.) according to your consideration.

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