Review



# The Latest Trends in Cryopreservation of Fungi

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*Review*

# The Latest Trends in Cryopreservation of Fungi

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**Abstract:** Fungi belong to the species with the highest biodiversity and high potential for use in biotechnology and industry. Therefore, their long-term quality conservation is very important and it is necessary to continuously develop new methods and improve existing methods. Currently, cryopreservation appears to be the best available technique for the preservation of fungi. This study summarises the latest trends and advances in fungal cryopreservation over the last 7 years, i.e. findings from scientific publications from 2017 to 2023 dealing with cryopreservation of both basidiomycetes and non-basidiomycete fungi. The work analyzed the studied organisms and the cryopreservation method used, focused on the use of new procedures and modifications of existing methods; summarized the conditions for successful cryopreservation, such as cryopreservation time and temperature, as well as the substrate and cryoprotectant used.

**Keywords:** basidiomycetes; non-basidiomycete fungi; cryopreservation; long/short-term storage; liquid nitrogene; cryoprotectant

## 1. Introduction

Various preservation methods such as subculturing, preservation under mineral oil, storage in sterile water and drying or freeze-drying cultures have been utilized for preservation of fungi in the past decay. However, these methods have many disadvantages and some of them are inappropriate for basidiomycetous fungi. Additionally, to preserve optimal long-term viability and genetic stability of the strains, it is essential to keep the strains in a metabolically inactive state. Currently, the preferred method is cryopreservation, the goal of which is the conserve of viable organisms and their genetic basis while preserving the ability to regenerate and limiting genetic changes as much as possible. The term cryopreservation generally refers to the preservation of organisms at temperatures lower than -20 °C, most often at temperatures from -70 °C to -150 °C in deep-freezing boxes or at -196 °C in liquid nitrogen or its vapors [1,2]. Cryopreservation includes the processes of freezing, preservation and thawing to a temperature optimal for the growth and reproduction of the organism, the implementation of which plays a key role in the viability of biological material. Inert materials are often used as carriers and protective media seems to be mostly necessary. Cryopreservation of some groups of microorganisms (primarily bacteria) in liquid nitrogen is easy with a high probability of culture survival. For other groups (some species of fungi and fungus-like organisms), further research and optimization of appropriate protocols for safe long-term storage is needed.

Widely used and the best cryopreservation method suitable for fungi is storage in liquid nitrogen [3]. Prepared culture samples are frozen directly or gradually, usually in a programmable device according to specific protocols, different for different groups of microorganisms, and then placed in a container with liquid nitrogen.

Already from the middle of the last century until now, various protocols for cryopreservation using liquid nitrogen have been published. The first works described a method where agar blocks immersed in cryoprotectant were used as carriers of fungal mycelium [4,5], followed by the straw technique using agar miniblocks in polypropylene straws (developed by Elliot [6], improved by Stalpers et al. [7] and modified by Hoffmann [8] and Homolka et al. [9]). A new method of cryopreservation using perlite as a carrier of fungal mycelium (perlite protocol) was developed by Homolka et al. in 2001 [10] and afterwards successfully verified on 442 strains of basidiomycetes [11].

Perlite is a unique aluminosilicate volcanic mineral that can retain water and release it as needed. Perlite protocol for fungi cryopreservation is used by various laboratories around the world; it was further modified for the needs of their fungal strains by Sato et al. [12–14].

The development of the cryopreservation method for basidiomycetes, from the first beginnings of this method to a few years ago, has been summarized in detail by some authors [15,16]. The presented work focused on the latest findings, trends and advances published in this area both for basidiomycetes and non-basidiomycete fungi and collected this information from 2017 to the present.

## 2. Studied Genera and Groups of Cryopreserved Fungi

Recently, researchers in the area of the cryopreservation of fungi have focused on both basidiomycets and non-basidiomycete fungi. Since basidiomycetes are harder to cryoconserve than other fungal groups producing abundant spores, a greater number of scientific works have paid attention to non-basidiomycete fungi. Of the 23 papers studied, 9 dealt with the study of the cryopreservation process in basidiomycetes, 13 in non-basidiomycete species and in one paper was tested both basidiomycete and non-basidiomycete species (Table 1). A total of 63 genera of fungi (29 genera of basidiomycetes and 34 genera of non-basidiomycete fungi) have been studied in the aforementioned publications from 2017 to 2023 (Table 2).

**Table 1.** Cryopreservation of fungi – overview of published papers in 2017-2023.

| Year | Organisms Studied* | Group of Fungi | Reference | DOI |
|---|---|---|---|---|
| 2017 | N | Entomopathogenic fungi (*Ascomycetes*) | [21] | http://dx.doi.org/10.1016/j.funbio.2017.07.007 |
| | B | Ectomycorrhizal fungi | [17] | http://dx.doi.org/10.1007/s00572-017-0770-3 |
| | N | Agriculturally important fungi (*Ascomycetes*) | [31] | - |
| 2018 | N | Edible and medicinal fungi *(Ascomycetes)* | [33] | https://doi.org/10.1007/s11274-018-2550-4 |
| | N and B | Phytopathogenic fungi (*Ascomycetes, Basidiomycetes)* | [24] | https://doi.org/10.1016/j.mimet.2018.04.007 |
| | N | Entomopathogenic fungi (*Ascomycetes*) | [23] | https://doi.org/10.1063/1.5064165 |
| | B | Edible and medicinal fungi | [18] | https://doi.org/10.1016/j.bjm.2017.08.003 |

| | | | | |
|---|---|---|---|---|
| 2019 | B | Edible, medicinal and biotechnologicaly important fungi | [36] | http://doi.org/10.1088/1755-1315/308/1/012069 |
| | N | Arbuscullar mycorrhizal fungi (*Glomeromycetes*) | [26] | https://doi.org/10.1016/j.apsoil.2019.04.020 |
| | B | Edible and medicinal fungi | [20] | https://doi.org/10.1007/s42770-019-00063-9 |
| | B | Ectomycorrhizal fungi | [12] | https://doi.org/10.1080/00275514.2018.1520035 |
| 2020 | N | Pathogenic fungi *(Oomycotetes)* | [39] | https://doi.org/10.1016/j.funbio.2020.04.005 |
| | B | Ectomycorrhizal fungi | [13] | https://doi.org/10.1016/j.funbio.2020.05.002 |
| | B | Edible and medicinal fungi | [19] | https://doi.org/10.1016/j.mimet.2020.106030 |
| 2021 | N | Thermophilic fungi (*Ascomycetes*, *Zygomycetes*) | [27] | https://doi.org/10.1016/j.cryobiol.2021.06.005 |
| | B | Ectomycorrhizal fungi | [14] | - |
| 2022 | B | Edible, medicinal and biotechnologicaly important fungi | [35] | https://doi.org/10.1016/j.mimet.2022.106491 |
| | N | Endophytic and medicinal fungi (A*scomycetes*) | [25] | https://doi.org/10.1007/s11274-022-03278-5 |
| | N | Anaerobic fungi (*Neocallimastigomycetes*) | [30] | https://doi.org/10.3389/fmicb.2022.978028 |

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|---|---|---|---|---|
| | N | Plant and fungal pathogenic fungi, industrially importat fungi (*Ascomycetes*) | [32] | https://doi.org/10.25303/1707rjbt65067 |
| 2023 | N | Human and animal pathogenic fungi (*Ascomycetes*) | [29] | https://doi.org/10.3390/jof9010034 |
| | N | Entomopathogenic fungi (*Ascomycetes*) | [22] | https://doi.org/10.1016/j.mimet.2023.106711 |
| | N | Entomopathogenic and medical fungi (*Ascomycetes*) | [34] | https://doi.org/10.1016/j.lwt.2022.114044 |

*B - Basidiomycetes, N - Non-basidiomycete fungi.

**Table 2.** Fungi tested in recent cryopreservation studies.

| Fungal genus | Basidiomycetes | Non-basidiomycete fungi | references |
|---|---|---|---|
| *Agaricus* | + | | 18, 19, 20, 36 |
| *Agrocybe* | + | | 36 |
| *Alternaria* | | + | 24 |
| *Amanita* | + | | 12, 13, 14 |
| *Anaeromyces* | | + | 30 |
| *Akanthomyces* | | + | 22 |
| *Aseroe* | + | | 12 |
| *Aspergillus* | | + | 32 |
| *Auricularia* | + | | 36 |
| *Bauveria* | | + | 22, 24 |
| *Botrytis* | | + | 21, 24 |
| *Claroideoglomus* | | + | 26 |
| *Collybia* | + | | 36 |
| *Coprinus* | + | | 36 |
| *Cordyceps* | | + | 22, 33, 34 |
| *Funneliformis* | | + | 26 |
| *Fusarium* | | + | 24 |
| *Ganoderma* | + | | 36 |
| *Hebeloma* | + | | 12,14, 17 |
| *Hirsutella* | | + | 21, 22 |
| *Humicola* | | + | 27 |
| *Kobayasia* | + | | 12, 13, 14 |

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|---|---|---|---|
| *Isaria* | | + | 21 |
| *Laccaria* | + | | 17 |
| *Lactarius* | + | | 12, 13, 14 |
| *Lecanicillium* | | + | 21 |
| *Lentinula (Lentinus)* | + | | 19, 35, 36 |
| *Lyophyllum* | + | | 12, 14 |
| *Macrocybe* | + | | 13 |
| *Malbranchea* | | + | 27 |
| *Melanocarpus* | | + | 27 |
| *Metarhizum* | | + | 21, 22, 23 |
| *Mycothermus* | | + | 27 |
| *Neocallimastix* | | + | 30 |
| *Nigrospora* | | + | 25 |
| *Nodulisporium* | | + | 25 |
| *Penicillium* | | + | 25 |
| *Phallus* | + | | 12, 14 |
| *Phanerochaete* | + | | 36 |
| *Phomopsis* | | + | 25 |
| *Pisolithus* | + | | 17 |
| *Pleurotus* | + | | 19, 36 |
| *Rhizoctonia* | + | | 24 |
| *Rhizomucor* | | + | 27 |
| *Rhizophagus* | | + | 26 |
| *Rhizopogon* | + | | 12, 14, 17 |
| *Russula* | + | | 36 |
| *Saprolegnia* | | + | 39 |
| *Sarcodon* | + | | 12, 13, 14 |
| *Schizophyllum* | + | | 19 |
| *Sclerotinia* | | + | 24 |
| *Sporothrix* | | + | 29 |
| *Suillus* | + | | 12, 14, 17 |
| *Thanatephorus* | + | | 24 |
| *Thielavopsis* | | + | 24 |
| *Thermomyces* | | + | 27 |
| *Thermothelomyces* | | + | 27 |
| *Thermothielavioides* | | + | 27 |
| *Trametes* | + | | 36 |
| *Trichocladium* | | + | 27 |
| *Trichoderma* | | + | 24, 31 |
| *Tricholoma* | + | | 12, 13, 14 |

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| | | |
|---|---|---|
| *Waitea* | + | 24 |

In recent years, the focus has been mainly on ectomycorrhizal species of basidiomycetes [12–14,17], which are often more cryosensitive than other basidiomycetes and and thus harder to store at low temperatures; then on to medicinal and edible fungi [18–20]. The most often studied and cryopreserved basidiomycete genus was *Agaricus*, which is widely cultivated around the world, especially in the US, Brazil and in European countries [16] followed by another frequently studied genera such as *Amanita*, *Hebeloma*, *Kobayasia*, *Lactarius*, *Lentinula*, *Pleurotus*, *Rhizopogon*, *Sarcodon*, *Suillus* and *Tricholoma* (see Table 2). These genera are the most cultivated fungi due to their biotechnological and nutritional potential.

Among non-basidiomycete groups of fungi, *Ascomycetes* were the most frequently studied and cryopreserved, followed by *Zygomycetes* and *Oomycetes*. The papers studied paid attention to entomopathogenic fungi [21–23], phytopathogenic species [24], medicinal fungi [25], arbuscular mycorrhizal fungi [26], thermophilic fungi [27], dermatophytes [28] and other human pathogenic fungi [29], anaerobic fungi [30] etc. (see Table 1). The most frequently studied genera among these fungi was *Cordyceps* and *Metarhizum*, followed by *Bauveria*, *Botrytis*, *Hirsutella* or *Trichoderma*; i.e. fungi used in agriculture to protect crops from pests, bacterial and fungal diseases, fungi caused human or plant diseases or fungi with medicinal and nutritional potential [31–34]. All these areas of human activity have gained importance in recent years, therefore methods of long-term cryopreservation of relevant fungal organisms are also of interest to researchers.

## 3. Current Methods of Cryopreservation of Fungi

Many studies from recent years compare cryopreservation methods at ultra-low temperatures with methods based on the preservation of tested organisms at higher temperatures, most often at +4 °C or at room temperature. However, our review focuses on cryopreservation methods, i.e. the preservation of fungi at temperatures of -20 °C and lower, therefore the following information relates only to these methods. References and information regarding the use of lyophilization methods for the preservation of fungal organisms were also not included in this review.

Basidiomycete cryopreservation methods often use various types of fungal mycelium carriers, among which wheat grains have been most frequently mentioned in recent years (Table 3). Typically, sterile wheat grains were inoculated on agar discs containing mycelium and kept at 25°C until most of the grains in the tube were colonized with mycelium. Mycelium-grown grains were then used for the freezing process in cryotubes or closed polypropylene straws [18–20,35]. Some papers describe cryopreservation without the use of a carrier, where agar discs with grown mycelium were frozen directly in the medium in ampoules or cryotubes [17,18,36]. A method based on direct mycelial growth in cryotubes was applied to almost 100 isolates of ectomycorrhizal fungi already in 2013 [37], when Crahay and co-workers developed a cryopreservation protocol called the “Cryovial Protocol”. They subsequently followed up on their previous research and demonstrated that this method does not affect the physiological stability of the frozen isolates or their ability to colonize plant roots and the genetic stability of the isolates is also preserved. Therefore, this method can be successfully used in the cryopreservation of ectomycorrhizal fungi [17]. Nowadays, cryopreservation studies are increasingly concerned with verifying the genetic stability of organisms [17,18], whereas earlier work focused more on the effect of cryopreservation on the physiological and morphological properties of frozen organisms.

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**Table 3.** Methods of cryopreservation of basidiomycetes.

| Method (cryopreserved material) | Cryopreservation temperature | Cryoprotectants | Storage | | Reference |
|---|---|---|---|---|---|
| | | | Short time (h, w, m, y)* | Long time (y)* | |
| cryovial protocol | -130 °C | glycerol 10% | | 2 | [17] |
| wheat grains | -80 °C | glycerol 20% | 6 m | 2, 3, 4, 5 | [24] |
| wheat grains, agar discs with mycelium | -20 °C, -75 °C, | sucrose 10% - 45% | 1 y | 2 | [18] |
| agar discs with mycelium | -80 °C | glycerol 5%, trehalose 10% | 48 h<br>2 w<br>1 y | over 3 | [36] |
| wheat or rye grains, agar discs with mycelium | -70 °C | glycerol 5% | | 5 | [20] |
| modified perlite protocol | -80 °C | combinations of DMSO**<br>5 or 10 %<br>Glycerol<br>5 or 12% | 2 w<br>6 m<br>1 y | | [12] |

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|---|---|---|---|---|---|
| | | trehalose<br>5 or 10% | | | |
| vermiculit protocol,<br>agar discs with mycelium | -80 °C | mixture of DMSO or gycerol 5% and trehalose 10% | 1-6 w<br>1 y | | [13] |
| wheat grains | -80 °C | glucose 4% | | 2, 5 | [19] |
| modified perlite protocol | -80 °C<br>-170 °C | glycerol 5% | | 5, 6 | [14] |
| wheat grains,<br>agar disks with mycelium | -86 °C | DMSO<br>1.5 - 2.5%<br>glycerol<br>3.5 - 5.5 %<br>sucrose<br>25 - 35%<br>glucose<br>5 - 9% | 1, 6 m<br>1 y | 2 | [35] |

*h - hours, w - weeks, m - months, y - years; short time = up to 1 year, long time = over 1 year **DMSO - dimethyl sulfoxide.

A suitable carrier currently used for cryopreservation of basidiomycetes is perlite. The original method of Homolka [10,11], the so-called „Homolka perlit protocol", was adapted by Sato et al. to the needs of the ectomycorrhizal fungi studied by them. They had already modified the perlite protocol in 2012 [38] by adding cryoprotectant just before freezing instead of culturing the mycelia with cryoprotectant, thus significantly reducing the time of action of the cryoprotectant on the mycelia. However, this procedure needed to be verified on a larger set of fungi, which they published in 2019 [12]. They published a paper in which they tested 111 strains of 38 basidiomycete species of varying cryo-sensitivity and improved this protocol by optimizing the cryoprotectants and testing its different concentrations and combinations. This work confirmed the suitability of the modified perlite method for cryopreservation of ectomycorrhizal basidiomycetes. Subsequently, Sato et al. published a paper where they tested another carrier in the cryopreservation of basidiomycetes,

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namely vermiculite [13]. This has similar properties to perlite and was used in the cryopreservation of 12 ectomycorrhizal strains of basidiomycetes that showed poorer results using previous methods, both the "Homolka perlite protocol" and the "Modified perlite protocol". Using vermiculite, all studied cultures were viable after 1 year of cryopreservation at -80 °C, so this method can be successfully used for cryopreservation of cryosensitive ectomycorrhizal basidiomycetes.
In 2021, Sato et al. tested a set of 105 species of basidiomycetes, including cryosensitive ectomycorrhizal species, according to Homolka perlit protocol and stored them for five years in freezer at -80 °C and 25 of them (cryosensitive species) they stored for six years in a tank of liquid nitrogen in its vapors [14]. More than 80% cultures showed good recovery after five years of storage at -80°C. Surprisingly, some strains showed poor viability when stored at - 80 °C, but had a better recovery rate after storage at -170 °C. This suggests that cryopreservation in vapor-phase liguid nitrogen is more effective for long term storage of cryosensitive fungi.

In contrast to basidiomycetes, most non-basidiomycete fungi produce abundant spores, which makes their preservation easier and a wider range of methods are available. The spores of these fungi can be easily preserved in water or mineral oil at normal temperatures, but increasingly methods using ultra-low temperatures (-70 °C to -196 °C) are also being used for these organisms (Table 4). In addition to viability, the main aim is to maintain the genetic stability of the organisms and to minimise any changes in these organisms during storage. As in the case of basidiomycetes, mycelial carriers (wheat grains) are used for non-basidiomycete fungi [25], but to a lesser extent, and more often the mycelium or spore suspension is frozen directly [e.g. 27,39 etc.]. Some papers, however, mention the use of less common carriers or substrates in the cryopreservation of nonbasidiomycete fungi; for example, Maxime at al. described an interesting protocol using pieces of filter paper as a carrier for mycelium and conidia [32], and Püschel et al. presented a peat-based substrate in the cryopreservation of arbuscular mycobacterial fungi [26]. Preservation in liquid nitrogen has also found its application here. Scientific work in recent years has shown that, in contrast to basidiomycetes, cryopreservation of non-basidiomycete species does not require gradual and controlled freezing, but samples can be stored directly at a suitable temperature [25,31].

**Table 4.** Methods of cryopreservation of non-basidiomycete fungi.

| Method (cryopreserved material) | Cryopreservation temperature | Cryoprotectants | Storage | | Reference |
|---|---|---|---|---|---|
| | | | Short time (h, w, m, y)* | Long time (y)* | |
| agar discs with mycelium and spores | -70 °C , -196 °C | glycerol 10 % | | 2 | [21] |
| agar discs with mycelium and spores, cultures on agar slants | -80 °C, -196 °C | glycerol 10 % | | 2 | [31] |

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| | | | | | |
|---|---|---|---|---|---|
| agar disks with mycelium and spores, spore suspension | -20 °C, -80 °C | skim milk, peptone 6 % | 24 h | | [33] |
| wheat grains, agar discs with mycelium and spores | -80 °C | glycerol 20 % | 6 m | 2, 3, 4, 5 | [24] |
| spore and hyphae suspension | -80 °C | glycerol 10 %, mixture of glycerol 10 % and trehalose 5 % | 1, 14, 30 d | | [23] |
| Peat - based substrate | -20 °C | - | | 4.6 | [26] |
| agar disks with mycelium and spores | -80°C | glycerol 10 % | 3, 6, 9, 12 m | | [39] |
| agar disks with mycelium and spores, spore suspension | -20 °C | glycerol 15 % | | 5 | [27] |
| agar disks with mycelium and spores | -80°C | glycerol 30 % | 6, 12 m | 1.5 2 | [29] |

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|---|---|---|---|---|---|
| wheat grains, agar disks with mycelium and spores | -80°C | sucrose 15 % | 1-6 m | | [25] |
| liquid culture | -80 °C, -196 °C | ethylene glycol, glycerol | 1w<br>3, 6, 9 m<br>1 y | | [30] |
| mycelia and spores on filter paper, liquid culture | -20°C, -80°C | glycerol, DMSO** | 1 m | | [32] |
| agar disks with mycelium and spores | -70 °C, -196 °C | - | | 3.8 - 8.2 | [22] |
| mycelium in larva body and fruit body | -20 °C, -80 °C, -196 °C | ethanol/water solution 50 % | 8 w | | [34] |

*h - hours, w - weeks, m - months, y - years; short time = up to 1 year, long time = over 1 year **DMSO - dimethyl sulfoxide.

**4. Cryopreservation Temperature for Fungi**

The storage temperature is one of the most important parameters of the cryopreservation process that affects the success of survival. From published studies, it is clear that most basidiomycetes have been stored at temperatures of -20 °C to -130 °C in a freezer; in liquid nitrogen at -196 °C or in its vapour at -170 °C (see Table 3). For successful cryopreservation, storage temperatures of -70 °C or -80 °C were most commonly used [e.g. 20,12,13,14,19, 20,36], rarely -86 °C [35]. On the other hand, the higher temperature of -20 °C was considered ineffective in some cases [18] because of the high variability of results and reduced mycelial viability. The cultures were mostly frozen gradually at a rate of 1 degree per min [e.g. 12,13, 17,35] or directly stored at the appropriate temperature [18,19]. Ilyas and Soeka [36] added 6-24 h of acclimatization of cultures at 4 °C before direct storage of fungi at -80 °C; Sato took a similar step by leaving the strains in the freezer at 5 °C for 48 h [12]. Crahay [17] published successful results of cryopreservation of ectomycorrhizal basidiomycetes using controlled freezing at different rates: from 20 °C to 4 °C at 8 °C per minute in the initial phase, then from 4 °C to -50 °C at 1 °C per minute and from -50 °C to -100 °C at 10 °C per minute followed by direct transfer to a freezer at 130 °C.

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Non-basidiomycete fungi were also usually stored at -70 °C or -80 °C [23,31,39], less frequently at -20 °C [27]; in liquid nitrogen at -196 °C or in its vapour phase [21,22] (see Table 4). As mentioned above, in most cases, samples of these fungi were stored directly at the relevant temperatures without the use of controlled stepwise freezing. However, Ayala-Zermeño [21] used pre-cooling and leaving the samples at 4 °C for 12 h before storage at -70 °C to allow diffusion of the cryoprotectant into the cells. Similarly, Vinzelj [30] placed samples on ice for 10 min and transferred them to -20 °C for 1 h, placed them at -80 °C for 24 h and then transferred them to liquid nitrogen for storage in either the gaseous or liquid phase.

## 5. Cryopreservation Period for Fungi

The studied papers focused on both long-term cryopreservation of basidiomycetes for more than 1 year [17,19,20] and short-term cryopreservation (from 1 week to 1 year) [12,13]. Most often, authors compared the results obtained from both short-term and long-term preservation of basidiomycetes [35,36] (see Table 3). The longest cryopreserved basidiomycete samples were for 5 years [19,20,24], and 6 years [14]. Sato et al. published in 2021 results from a 5-year cryopreservation of a large set of basidiomycetes (including cryosensitive ectomycorrhizal species) at -80 °C in a freezer and in liquid nitrogen vapor, where they were preserved for 6 years.

As with the basidiomycetes, recent studies of non-basidiomycete fungi have addressed both long-term and short-term preservation of the strains studied (see Table 4). Ayala-Zermeño [22] cryopreserved samples of entomopathogenic fungi at -70 °C and -196 °C for more than 8 years (8.2 years), and Berikten [27] and Lakshman [24] stored fungi for up to 5 years. On the other hand, there are studies that have tested the preservation of non-basidiomycete samples over a range of only days [23,33], weeks [34] or months [32] up to one year [30,39].

## 6. Cryoprotectants for Fungal Cryopreservation

The most commonly used cryoprotectant in the basidiomycetes is glycerol followed by dimethyl sulfoxide (DMSO), sucrose, trehalose or glucose (individually or in mixtures) in concentrations from 5% to 45% (see Table 3). Ilyas and Soeka [36] used a mixture of glycerol and trehalose for cryopreservation of 50 strains of basidiomycetes. They demonstrated that there was no loss of viability between cultures stored for 48 hours and more than 3 years at -80 °C. However, Zahri Júnior et al. [18] in cryopreservation of *Agaricus subrufescens* at -75 °C used 10% sucrose in the substrate and 15% sucrose in cryotubes with wheat grains as carriers. They also tested the absence of a cryoprotectants and found that when using a wheat grains carrier (wheat graine technique) the culture survived even without a cryoprotectant. In another work, they successfully used 4% glucose and wheat grain technique for cryopreservation of variosu edible and medicinal basidiomycetes and confirmed its suitability for these species [19]. The role of cryoprotectant (5% glycerol) in cryopreservation of *Agaricus subrufescens* was also tested by Tanaka et al. [20]. They studied the effect of cryoprotectant added to the substrate and/or cryotubes on the viability after cryopreservation of samples depending on the inoculum used and the consistency of the regeneration culture medium. They found that agar-based substrates with or without the addition of glycerol were not effective in maintaining mycelial viability regardless of the consistency of the regeneration culture medium. In contrast, the use of grains with or without the addition of glycerol was effective for cryopreservation of *Agaricus* for 5 years. This viability was higher when a semi-solid consistency of the regeneration culture medium was used. Sato at al. used Homolka perlite protocol with 5% glycerol for cryopreservation of a large set of fungi [20]. In their modified perlite protocol, they tested other cryopreservatives such as dimethyl sulfoxide, trehalose, etc. in different concentrations and their various combinations and also investigated the potential toxicity of cryopreservatives on some cryosensitive fungal organisms. They found that both glycerol and dimethyl sulfoxide were suitable for ectomycorrhizal basidiomycetes, but dimethyl sulfoxide was less toxic to some strains than glycerol. Therefore, they modified Homolka's perlite protocol by adding glycerol before freezing instead of culturing samples with it. They also found that the addition of trehalose to glycerol or to dimethyl sulfoxide increased their cryoprotective effect.

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Also in case of non-basidiomycete fungi was glycerol (from 10 to 30%) the most frequently studied cryoprotectant (see Table 4). Sari et al [23] used a mixture of 10% glycerol and trehalose, also some another cryoprotectants like sucrose [25], dimethyl sulfoxide [32], ethanol [34], skim milk or peptone [33] were tested recetly.

## 7. Recovery of the Cryopreserved Fungal Samples

In the cryopreservation process of fungal samples, the thawing temperature and the culture medium used in the recovery after freezing are important parameters. Most papers in the field of cryopreservation of basidiomycetes report a similar procedure for recovery of cryopreserved specimens: usually cryovials were directly immersed in a water bath at 30-38 °C for 2-3 minutes, [12–14,17,36] or for 15 minutes [18–20,35]. The culture medium for recovery after cryopreservation (solid, semi-solid or liquid) is based on the medium in which the fungus originally grew before freezing. The most commonly used medium for recovery was potato dextrose agar (PDA) [18,19,36] or malt extract agar medium (MEA) [20,35], where the fungi were cultured at 25-27 °C.

Some non-basidiomycete fungi, like basidiomycetes, recovered after freezing by thawing in a water bath at 30 to 37 °C [21,25,31], or at room temperature [39], for a several minutes. Samples were then placed in Petri dishes usually with potato dextrose agar [24,25,31] or with Sabouraud dextrose agar with yeast extract [21,23]. It is clear from the recent work studied that because non-basidiomycete species include a very diverse group of fungi both taxonomically (ascomycetes, oomycetes, etc.) and in terms of life strategies or ecology (e.g. thermophilic fungi, symbiotic or parasitic fungi), it is usually not possible to use a uniform recovery protocol for thawing cryopreserved samples. Specific modified recovery procedures have been published, for example, for some pathogenic species [29] or for anaerobic fungi [30].

## 8. Conclusion and Future Perspectives

Cryopreservation of fungi is a long-term preservation method that allows storage of microorganisms at low or ultra-low temperatures while maintaining their viability, purity and genetic stability [40]. Scientific papers published in the last 7 years have clearly shown an increasing interest in the use of this method in both basidiomycetes and non-basidiomyce fungi.

In particular, difficult-to-cryopreserve groups of fungi, such as ectomycorrhizal basidiomycetes, as well as edible and medicinal mushrooms, which are becoming increasingly important in terms of nutrition and human health, are coming to the fore; furthermore, entomopathogenic and phytopathogenic fungi as well as human pathogenic fungi. From this point of view, it is not surprising that e.g. *Agaricus*, *Cordiceps*, *Lentinula*, *Metarhizum*, *Pleurotus or Trichoderma*, belonging to important fungi in terms of biotechnological use, nutrition, agriculture, health and other areas of human research, have recently become among most often cryopreserved genera.

Both basidiomycetes and non-basidiomycete fungi were usually cryopreserved at temperatures around -70 °C to -80 °C, in liquid nitrogen at -196 °C or in its vapours at -130 °C. Storing at -20 °C has often proven to be less effective, therefore the trend is towards long-term storage at lower temperatures. Since basidiomycetes are more difficult to cryopreserve than non-basidiomycetes species, current methods clearly point to the use of controlled, gradual freezing in basidiomycetes, whereas non-basidiomycetes can be frozen directly.

The maximum retention period in the publications studied was 5 - 6 years for basidiomycetes and 8.2 years for non-basidiomycete fungi. Cereal grains and perlite in basidiomycetes proved to be the best mycelium carriers, but agar-based substrates are still used for long-term cryopreservation. The use of perlite, modification of current perlite protocols and the use of other similar carriers such as vermiculite is the most significant trend in recent years in the cryopreservation of basidiomycetes. Non-basidiomycete fungi, which form spores easily, are usually frozen as spore suspensions or as agar substrates with spore-rich mycelia; carriers in the form of e.g. cereal grains are also used in this group, but less frequently.

In cryopreservation of basidiomycetes and non-basidiomycete fungi, the most effective cryoprotectant was glycerol, which was used alone or in mixtures with other cryoprotectants such as

dimethyl sulfoxide, sucrose, trehalose, glucose. Many different aspects play a role in the process of recovery of cryopreserved fungi, and due to the diversity of the fungal kingdom, this area is still little explored. Mycelium viability, morphological and genetic stability are important parameters of analysis of fungal samples after cryopreservation and an indicator of cryopreservation efficiency, therefore the creation of optimal recovery protocols for different species or groups of fungi is a challenge for future research in this area.

In conclusion, it is necessary to continue the study of cryopreservation techniques and the development of new effective methods or modifications of existing protocols with the aim of their use for the conservation and cryopreservation of fungi that are important both from the point of view of preserving the gene pool and diversity, as well as with the aim of using these species in various areas of human activity, especially in the field of nutrition, medicine, agriculture and biotechnology.

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## References

1. Smith, D.; Ryan, M. Implementing best practices and validation of cryopreservation techniques for microorganisms. *Sci. World J.* ***2012***, Article ID 805659, 9 p. DOI: 10.1100/2012/805659
2. Homolka, L. Methods of Cryopreservation in Fungi. In Laboratory Protocols in Fungal Biology. Fungal Biology. Gupta, V., Tuohy, M., Ayyachamy, M., Turner, K., O'Donovan, A., Eds.; Springer, New York, NY. 2013; 2-10. DOI:10.1007/978-1-4614-2356-0_2
3. Smith, D. The use of cryopreservation in the ex-situ conservation of fungi Cryo Lett. 1998, 19, 79-90.
4. Hwang, S.W. Effects of ultra-low temperatures on the viability of selected fungus strains. *Mycologia* 1960, *52*, 527-529.
5. Hwang, S.-W. Long-term preservation of fungus cultures with liquid nitrogen refrigeration. *Appl. Microbiol.* 1966, *14*, 784-788.
6. Elliott, T.J. Alternative ampoule for storing fungal cultures in liquid nitrogen. *Trans. Br. Mycol. Soc.* 1976, *67*, 545-546.
7. Stalpers, J.A.; de Hoog, A.; Vlug, I.J. Improvement of the straw technique for the preservation of fungi in liquid nitrogen. *Mycologia* 1987, *79*, 82-89.
8. Hoffmann, P. Cryopreservation of fungi. *World J. Microbiol. Biotechnol.* 1991, *7*, 92-94.
9. Homolka, L.; Lisá, L.; Nerud, F. Viability of basidiomycete strains after cryopreservation: comparison of two different freezing protocols. *Folia Microbiol.* 2003, *48*, 219-226.
10. Homolka, L.; Lisá, L.; Eichlerová, I.; Nerud, F. Cryopreservation of basidiomycete strains using perlite. *J. Microbiol. Meth.* 2001, *47*, 307-313.
11. Homolka, L.; Lisá, L.; Nerud, F. Basidiomycetes cryopreservation on perlite: evaluation of a new method. *Cryobiology*, 2006, *52*, 446-453.
12. Sato, M.; Inaba, S.; Sukenobe, J.; Sasaki, T.; Inoue, R.; Noguchi, M.; Nakagiri, A. A modified perlite protocol with a mixed dimethyl sulfoxide and trehalose cryoprotectant improves the viability of frozen cultures of ectomycorrhizal basidiomycetes. *Mycologia* 2019, *111*, 161-176. DOI: 10.1080/00275514.2018.1520035
13. Sato, M.; Inaba, S.; Noguchi, M.; Nakagiri, A. Vermiculite as a culture substrate greatly improves the viability of frozen cultures of ectomycorrhizal basidiomycetes. *Fungal Biol.* 2020, *124*, 742-751. DOI: 10.1016/j.funbio.2020.05.002
14. Sato, M.; Inaba, S.; Noguchi, M.; Okazaki, N.; Nakagiri, A. Long-term cryopreservation of basidiomycete strains with Homolka's perlite protocol followed by storage in an ultra-low-temperature freezer and vapor-phase liquid nitrogen tank. *Microb. Resour. Syst.* 2021, *37*, 13-20.
15. Homolka, L. Preservation of live cultures of basidiomycetes - recent methods. *Fungal Biol.* 2014, *118*, 107-125. DOI: 10.1016/j.funbio.2013.12.002

16. Linde, G.A.; Luciani, A.; Lopes, A.D.; Valle, J.S.D.; Colauto, N.B. Long-term cryopreservation of basidiomycetes. *Braz. J. Microbiol.* 2018, *49*, 220-231. DOI: 10.1016/j.bjm.2017.08.004
17. Crahay, Charlotte; Munaut, Françoise; Colpaert, Jan V.; Huret, Stéphanie; Declerck, Stephan. Genetic stability of ectomycorrhizal fungi is not affected by cryopreservation at −130 °C or cold storage with repeated sub-cultivations over a period of 2 years. Mycorrhiza 2017, 27, 595-601. DOI: 10.1007/s00572-017-0770-3
18. Zahri Júnior, L.L..; Lopes, A.D.; Cordeiro, F.A.; Colla, I.M.; Bertéli, M.B.D.; Valle, J.S.D.; Linde, G.A.; Colauto, N.B. Cryopreservation at -75°C of Agaricus subrufescens on wheat grains with sucrose. *Braz. J. Microbiol.* 2018, *49*(2), 370-377. DOI: 10.1016/j.bjm.2017.08.003
19. Zaghi Junior, L.L.; Bertéli, M.B.D.; de Freitas, J.D.S.; de Oliveira Filho, O.B.Q.; Lopes, A.D.; Ruiz, S.P.; do Valle, J.S.; Linde, G.A.; Colauto, N.B. Five-year cryopreservation at -80 °C of edible and medicinal basidiomycetes by wheat grain technique. *J. Microbiol. Meth.* 2020, *176*, 106030. DOI: 10.1016/j.mimet.2020.106030
20. Tanaka, H.S.; Bertéli, M.B.D.; Cordeiro, F.A.; Lopes, A.D.; do Valle, J.S.; Linde, G.A.; Colauto, N.B. Semisolid culture medium improves mycelial recovery of Agaricus subrufescens cryopreserved in cereal grains. *Braz. J. Microbiol.* 2019, *50*, 527-532. DOI: 10.1007/s42770-019-00063-9
21. Ayala-Zermeño, M.A.; Gallou, A.; Berlanga-Padilla, A.M.; Andrade-Michel, G.Y.; Rodríguez-Rodríguez, J.C.; Arredondo-Bernal, H.C.; Montesinos-Matías, R. Viability, purity, and genetic stability of entomopathogenic fungi species using different preservation methods. Fungal Biol. 2017, 121 (11) 920-928. DOI: 10.1016/j.funbio.2017.07.007
22. Ayala-Zermeño, M.A.; Berlanga-Padilla, A.M.; Regla-Márquez, C.F.; Lino-López, G.J.; Muñiz-Paredes, F.; Montesinos-Matías, R.; Sánchez-González, J.A. Long-term preservation and genetic stability of entomopathogenic fungal species. *J. Microbiol. Meth.* 2023, *208*, 106711. DOI: 10.1016/j.mimet.2023.106711
23. Sari, D.C.A.F.; Oetari, A.; Sjamsuridzal, W. Viability of Metarhizium majus UICC 295 after preservation by freezing at -80°C. *AIP Conf. Proc.* 2018, *2023*, 020168. DOI: 10.1063/1.5064165
24. Lakshman, D.K.; Singh, V.; Camacho, M.E. Long-term cryopreservation of non-spore-forming fungi in Microbank™ beads for plant pathological investigations. *J. Microbiol. Meth.* 2018, 148, 120-126. DOI: 10.1016/j.mimet.2018.04.007
25. Liu, Q.; Wang, F.; Liu, K.; Dong, C. Influence of Strain Preservation Methods on Fruiting Body Growth and Metabolite Production by the Medicinal Mushroom Cordyceps militaris (Ascomycetes). *Int. J. Med. Mushrooms* 2018, *20*, 1003-1011. DOI: 10.1615/IntJMedMushrooms.2018028582
26. Püschel, D.; Kolaříková, Z.; Šmilauer, P.; Rydlová, J. Survival and long-term infectivity of arbuscular mycorrhizal fungi in peat-based substrates stored under different temperature regimes. *App. Soil Ecol.* 2019, *140*, 98-107. DOI: 10.1016/j.apsoil.2019.04.020
27. Berikten, D. Survival of thermophilic fungi in various preservation methods: A comparative study. *Cryobiology* 2021, *101*, 38-43. DOI: 10.1016/j.cryobiol.2021.06.005
28. Jesús García-Martínez, Daniel López Lacomba, and Angel Castaño Pascual. Evaluation of a Method for Long-Term Cryopreservation of Fungal Strains. Biopreserv. Biobank. 2018, 16, 128-137. DOI: 10.1089/bio.2017.0101
29. de Souza Rabello, V.B.; Corrêa-Moreira, D.; Santos, C.; Abreu Pinto, T.C.; Procopio-Azevedo, A.C.; Boechat, J.; Coelho, R.A.; Almeida-Paes, R.; Costa, G.; Lima, N.; Zancopé-Oliveira, R.M.; Marques Evangelista Oliveira, M. Preservation Methods in Isolates of *Sporothrix* Characterized by Polyphasic Approach. *J.Fungi* 2023, *9*, 34. DOI: 10.3390/jof9010034
30. Vinzelj, J.; Joshi, A.; Young, D.; Begovic, L.; Peer, N.; Mosberger, L.; Luedi, K.C.S.; Insam, H.; Flad, V.; Nagler, M.; Podmirseg, S.M. No time to die: Comparative study on preservation protocols for anaerobic fungi. *Front. Microbiol.* 2022, *13*, 978028. DOI: 10.3389/fmicb.2022.978028
31. Iqbal, S.; Ashfaq, M.; Malik, A.M.; Inam-ulhaq, Khan, K.S.; Mathews, P. Isolation, preservation and revival of Trichoderma viride in culture media. J. Entomol. Zool. Stud. 2017, 5, 1640–1646.
32. Mehreb, M.; Rachel, M.; John, M.; Rawad, H.; Cijo, V. Assessment of Simple Methods to preserve Pure Cultures of *Aspergillus Versicolor* and *Aspergillus Niger*. *Res. J. Biotechnol.* 2022, *17*, 65-67. DOI: 10.25303/1707rjbt65067
33. Sun, H.; Hu, T.; Guo, Y. et al. Preservation affects the vegetative growth and fruiting body production of Cordyceps militaris. World J. Microbiol. Biotechnol. 2018, 34, 166. DOI: 10.1007/s11274-018-2550-4
34. Li, D.; Zhu, Z.; Sun, D.W. Effects of high-pressure freezing and deep-frozen storage on cell structure and quality of *Cordyceps sinensis*. *LWT-Food Sci. Technol.* 2023, *175*, 114044. DOI: 10.1016/j.lwt.2022.114044
35. Bertéli, M.B.D.; Pinheiro, C.R.; Philadelpho, B.O.; Otero, D.M.; Ribeiro, C.D.F.; de Souza, C.O.; Ferreira, E.S.; Ruiz, S.P.; do Valle, J.S.; Linde, G.A.; Colauto, N.B. Long-term cryopreservation of *Lentinus crinitus* strains by wheat grain technique. *J. Microbiol. Meth.*, 2022, *198*, 106491. DOI: 10.1016/j.mimet.2022.106491

36. Ilyas, M.; Soeka, Y.S. IOP Conf. Ser. Earth Environ. Sci. 2019, *308*, 012069. DOI: 10.1088/1755-1315/308/1/012069
37. Crahay, Ch.; Declerck, S.; Colpaert, J.V.; Pigeon, M.; Munaut, F. Viability of ectomycorrhizal fungi following cryopreservation, Fungal Biol., 2013, 117, 103-111. DOI: /10.1016/j.funbio.2012.12.003
38. Sato, M.; Sukenobe, J.; Nakagiri, A., Cryopreservation of cryosensitive basidiomycete cultures by application and modification of perlite protocol. Cryo-Lett. 2012, 33, 86-94.
39. Eszterbauer, E.; Hardy, T.; Rónai, Z.; Sipos, D.; Zsigmond, G. Cryopreservation of three Saprolegnia species (Oomycota): Preliminary evidence for the long-term archiving of water mould species. *Fungal Biol.* 2020, *124*, 682-687. DOI: 10.1016/j.funbio.2020.04.005
40. Castro-Rios, K.; Bermeo-Escobar, L.P. Methods for the culture conservation of edible and medicinal fungi. J. Microbiol. Biotechnol. Food Sci. 2021, 10, 620–625. DOI: 10.15414/jmbfs.2021.10.4.620-625