BRIEF REPORT

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Cryopreservation of Filamentous Micromycetes and Yeasts Using Perlite

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ABSTRACT. The viability, growth and morphology of 48 strains of *Ascomycota* (including 17 yeasts) and 20 strains of *Zygomycota* were determined after a 2-d and then after 1-year storage in liquid nitrogen using a new cryopreservation method with perlite as a particulate solid carrier. In case of *Ascomycota*, 45 strains (94 %) out of 48 survived both 2-d and 1-year storage in liquid nitrogen, respectively. In case of *Zygomycota*, all 20 strains survived both storage. In addition, 3 strains of *Basidiomycota* counted among yeasts were tested and all survived the 1 year storage. In all surviving cultures no negative effects of cryopreservation by this method have been observed after 1-year of storage in liquid nitrogen. The results indicate that the perlite protocol can be successfully used for cryopreservation of taxonomically different groups of fungi and also for fungi which failed to survive other routinely used preservation procedures.

A well-defined and taxonomically determined starting material is required for efficient research and commercial work in mycology; this is ensured by its safe storage. Repeated subculturing, which is frequently used as a routine method for preservation of fungi, is not very practical for storing large numbers of cultures. It is time-consuming, prone to contamination and does not prevent genetic and physiological changes during long-term maintenance. Various storage methods have been developed in order to eliminate these disadvantages. Besides lyophilization, the storage in liquid nitrogen (LN) has been considered the best preservation technique available for fungi, which seems to surpass all others in the ability to preserve genomic and phenotypic features (Smith 1998). It is a safe and reliable method for a long-term maintenance of most fungal species, especially those not amenable to freeze-drying. Agar blocks immersed in an appropriate cryoprotectant were originally used as carriers of fungal mycelium for the cryopreservation process (Hwang 1968). Then a useful straw technique with agar miniblocks for the preservation of fungi in LN was developed by Elliott (1976), improved by Stalpers *et al*. (1987) and modified by other authors (Hoffmann 1991; Homolka *et al*. 2003). A similar method using sealed polyethylene ampoules was described for the storage of yeasts (Hubálek and Kocková-Kratochvílová 1982), while a method using porous beads was successfully tested for cryopreservation of fungal spores (Chandler 1994). The cryopreservation process includes freezing and thawing and the protocol of these procedures plays an important part (Leef and Mazur 1978; Ryan *et al.* 2001). A comprehensive and detailed overview of the methods and results of cryopreservation of microorganisms, including fungi, was published by Hubálek (1996).

A new method of cryopreservation using perlite as a carrier of fungal mycelium (perlite protocol; PP) was developed in our laboratory (Homolka *et al*. 2001) and then successfully verified on 442 of basidiomycete strains (Homolka *et al*. 2006). Perlite is a unique aluminosilicate volcanic mineral holding and retaining substantial amounts of water, which can be released as needed. In this work we tested the possibility of using this method for cryopreservation of other different groups of fungi.

The fungi used (Table I), obtained from the *Culture Collection of Fungi, Department of Botany, Faculty of Science, Charles University* (CCF), and *Culture Collection of Yeasts, Department of Genetics and Microbiology, Faculty of Science, Charles University* (DMUP) Prague (Czechia), were maintained by serial transfers and kept on wort agar slants (wort 4° Balling, 1.5 % agar *Difco*) at 4 °C.

Perlite cryopreservation protocol (Homolka *et al*. 2001) was the following: Fungal cultures were grown directly in sterile plastic Nunc CryoTube Vials (1.8 mL) with 200 mg of perlite (Agroperlit, agricultural grade; *Keramik*, Czechia) moistened with 1 mL of wort (4° Balling) enriched with glycerol (final concentration 5 %; *Sigma*) as a cryoprotectant, sterilized by autoclaving, inoculated with an agar plug (diameter 6 mm) cut from the actively growing part of a colony on a Petri dish containing wort medium (wort 4° Bal-

Table I. Viability of individual strains after cryopreservation

Genus and species[a]	Strain no.	PP NSS[b]		Growth, mm[c]	
		2 d	1 year	before	after
Ascomycota					
Amblyosporium botrytis	CCF 2874	6	6	32 ± 5.0	30 ± 4.2
Arthrographis cuboidea	CCF 1128	6	6	31 ± 4.0	32 ± 3.6
Aspergillus carbonarius	CCF 2952	6	6	38 ± 5.6	35 ± 3.6
*Aureobasidium pullulans***	CCF 3129	6	6	26 ± 3.3	28 ± 4.2
	DMUP 1-1-9	6	6	26 ± 2.8	26 ± 3.5
Botryosphaeria stevensii	CCF 3445	6	6	29 ± 6.2	31 ± 4.3
Botrytis cinerea	CCF 917	6	6	49 ± 7.1	46 ± 6.7
Calcarisporium arbuscula	CCF 2721	5	6	11 ± 2.2	13 ± 3.6
*Candida pulcherrima**	DMUP 2-8-2	6	6	10 ± 3.5	10 ± 4.4
*utilis**	DMUP 2-10-1	6	6	10 ± 3.1	12 ± 4.8
Chaetomium globosum	CCF 2785	6	6	39 ± 5.8	35 ± 3.5
Chalaropsis thielavioides	CCF 3486	5	5	16 ± 1.9	15 ± 2.3
Cladosporium herbarum	CCF 3304	0	0	10 ± 2.8	0
Coniothyrium fuckelii	CCF 3178	6	6	25 ± 4.0	26 ± 5.5
Cylindrocarpostylus gregarius	CCF 2751	6	6	8 ± 1.5	9 ± 2.2
Dactylaria lanosa	CCF 2982	5	5	8 ± 1.6	6 ± 3.8
Dendryphion penicillatum	CCF 3190	6	6	26 ± 2.8	25 ± 2.1
Doratomyces stemonitis	CCF 3246	0	0	10 ± 4.0	0
*Endomyces magnusii**	DMUP 4-1-1	6	6	25 ± 3.2	25 ± 3.9
Esteya vermicola	CCF 3115	6	6	12 ± 3.1	11 ± 4.4
Exserohilum pedicellatum	CCF 2727	6	6	49 ± 6.5	51 ± 5.7
Fusarium solani	CCF 3181	6	6	35 ± 3.8	31 ± 6.2
*Geotrichum candidum**	DMUP 5-1-1	6	6	25 ± 2.1	22 ± 3.4
Humicola grisea	CCF 3257	6	6	30 ± 3.5	31 ± 4.6
Hypoxylon serpens	CCF 3047	5	5	11 ± 7.2	13 ± 6.1
*Kluyveromyces lactis**	DMUP 8-4-4	6	6	10 ± 4.2	10 ± 4.4
*Lipomyces starkeyi**	DMUP 24-1-1	6	6	10 ± 2.1	8 ± 3.5
Mycogone rosea	CCF 2918	6	6	35 ± 4.1	37 ± 8.0
Oidiodendron cerealis	CCF 1373	6	6	13 ± 3.1	12 ± 4.7
Ophiostoma quercus	CCF 2988	6	6	24 ± 2.4	22 ± 3.2
Phialophora melinii	CCF 3077	0	0	10 ± 2.2	0
*Saccharomyces cerevisiae**	DMUP 12-3-16	6	6	10 ± 1.5	11 ± 2.1
	DMUP 12-4-20	6	6	16 ± 1.8	15 ± 2.2
	DMUP 12-4-128	6	6	10 ± 1.6	10 ± 2.0
	DMUP 12-4-48	6	6	8 ± 2.3	9 ± 2.7
	DMUP 12-7-20	6	6	8 ± 2.1	8 ± 2.8
	DMUP 12-4-83	6	6	10 ± 2.4	8 ± 2.1
*Saccharomycopsis fibuligera**	DMUP 14-1-1	6	6	24 ± 2.0	22 ± 2.8
*Schizosaccharomyces pombe**	DMUP 15-2-2	6	6	12 ± 1.6	14 ± 2.1
*Schwanniomyces alluvius**	DMUP 16-1-1	6	6	10 ± 2.7	12 ± 2.1
Sordaria fimicola	CCF 3327	6	6	51 ± 6.6	48 ± 7.1
Spicellum roseum	CCF 3032	6	6	38 ± 5.7	35 ± 3.9
Stemphylium herbarum	CCF 3189	6	6	35 ± 4.4	32 ± 4.2
Torula herbarum	CCF 1370	6	6	20 ± 4.1	21 ± 2.9
Trichophaea abundans	CCF 3079	6	6	55 ± 2.3	52 ± 3.6
Virgaria nigra	CCF 2880	6	6	33 ± 2.5	34 ± 3.0
*Wickerhamia fluorescens**	DMUP 21-1-1	6	6	15 ± 1.8	14 ± 2.0
*Zygosaccharomyces rouxii**	DMUP 26-1-1	6	6	8 ± 2.8	8 ± 3.5
Zygomycota					
Absidia coerulea	CCF 440	6	6	72 ± 5.0	70 ± 7.2
Actinomucor elegans	CCF 2565	6	6	71 ± 6.1	71 ± 8.3
Backusella lamprospora	CCF 2414	6	6	55 ± 5.2	46 ± 6.3
Circinella umbellata	CCF 2971	6	6	52 ± 3.9	55 ± 4.4
Cunninghamella elegans	CCF 1585	6	6	56 ± 2.8	57 ± 3.3
Gongronella butleri	CCF 473	6	6	30 ± 3.4	32 ± 4.8
Linderina pennispora	CCF 1574	6	6	54 ± 5.1	47 ± 6.6

Mortierella alpina	CCF 2873	6	6	31 ± 5.0	33 ± 4.7
elongata	CCF 1143	6	6	25 ± 3.7	25 ± 3.6
exigua	CCF 2860	6	6	20 ± 2.2	20 ± 4.1
gamsii	CCF 2864	6	6	30 ± 3.2	33 ± 2.1
humilis	CCF 2654	6	6	29 ± 2.8	31 ± 3.9
parvispora	CCF 2867	6	6	26 ± 3.3	25 ± 3.1
Mucor mucedo	CCF 2660	6	6	17 ± 2.4	15 ± 3.2
wosnessenskii	CCF 2636	6	6	15 ± 1.6	14 ± 2.4
Mycotypha microspora	CCF 1876	6	6	22 ± 1.9	19 ± 2.6
Phycomyces nitens	CCF 3276	6	6	73 ± 6.6	68 ± 4.9
Rhizopus microsporus	CCF 1570	6	6	71 ± 5.1	72 ± 6.8
Syncephalastrum racemosum	CCF 1667	6	6	72 ± 3.9	67 ± 5.2
Umbelopsis isabellina	CCF 1098	6	6	55 ± 4.7	56 ± 6.2
Basidiomycota					
*Leucosporidium capsuligenum**	DMUP 23-1-1	6	6	25 ± 3.0	28 ± 4.4
*Cryptococcus albidus**	DMUP 3-1-1	6	6	10 ± 2.4	10 ± 1.8
*Sporidiobolus salmonicolor**	DMUP 25-2-1	6	6	15 ± 2.2	16 ± 3.4

[a]* – yeast, ** – yeast-like microorganism.
[b]Number of successfully surviving aliquots (out of 6) after 2 d or 1 year of storage in LN using PP.
[c]Diameter of a growth zone of the respective strain on a Petri dish after a 7- (yeast cultures) or 14-d (mycelial cultures) incubation before and after cryopreservation.

ling, 1.5 % agar Difco) and then incubated for 14 d at 24 °C. The cryovials with perlite overgrown by mycelium or yeast cells were frozen in a programmable freezer IceCube 1800 SyLab to –70 °C at a freezing rate of 1 K/min. They were then placed in LN in a Harsco TW-5K container. Thawing – reactivation of cultures – was carried out by transferring cryovials to a water bath (37 °C) until the ice was completely thawed. Prior to opening, the surface of cryovials was disinfected with ethanol. After thawing, the perlite particles overgrown with mycelium or cells were at least partially separated by shaking, the content of the cryovials (two parallels of each strain) was divided into three approximately equal aliquots and these were plated onto wort solid medium in Petri dishes using a small spoon and incubated at 24 °C for 7 (in case of yeasts) or 14 d (in case of mycelial strains). Strains exhibiting survival of 5 and/or 6 out of 6 separate aliquots were considered viable. Growth was measured as a diameter of a growth covered zone (mm) after a 7-d (in case of yeasts) or 14-d (in case of mycelial strains) incubation at 24 °C on wort agar medium in Petri dishes (diameter 100 mm) inoculated with perlite aliquots from cryovials before freezing and after reactivation. Six zones (3 aliquots from 2 cryotubes) were measured for each strain. The same procedure except for freezing and thawing was used for growth measurement of the control (the "before" column in Table I).

The viability and some other characteristics (growth, macro- and micromorphology) of 48 strains of *Ascomycota* (representing 42 species of 41 genera, including 17 strains of yeasts), 20 strains of *Zygomycota* (representing 20 species of 14 genera) and 3 strains of *Basidiomycota* counted among yeasts (representing 3 species of 3 genera) were determined after a 2-d and then after a 1-year storage in LN using PP. The tested set comprised a broad spectrum of strains with different systematic position, including both fast and slowly growing yeasts.

The recovery of the tested strains (counted as a percentage of surviving replicates) of individual species and their growth are summarized in Table I. The principal criterion of a successful recovery of fungal strains was the ability to retain the original growth and unchanged macro- and micromorphological characteristics.

Except for 3 relatively slowly growing ascomycete strains (*Cladosporium herbarum*, *Doratomyces stemonitis* and *Phialophora melinii*), all *Ascomycota* strains, including all yeasts, survived the storage in LN for 2 d and for 1 year successfully (in total 94 %). The failure of survival of *C. herbarum* (a relatively undemanding and resistant fungus) is surprising; however, the sensitivity to freezing is known to vary substantially even within one species. In case of *Zygomycota*, all 20 tested strains survived successfully both 2-d storage and 1-year storage in LN using PP. Similarly survived three *Basidiomycota* strains classed among yeasts which confirmed our previous results concerning basidiomycete strains (Homolka *et al*. 2003, 2006). Cryopreservation on perlite is especially suitable for strains not amenable to freeze-drying (lyophilization) which have had to be maintained by serial transfers, *e.g.*, *Botryosphaeria stevensii* CCF 3445, *Cylindrocarpostylus gregarius* CCF 2751, *Exserohilum pedicellatum* CCF 2727, *Hypoxylon serpens* CCF 3047, *Mycogone rosea* CCF 2918, *Stemphylium herbarum* CCF 3189, *Torula herbarum* CCF 1370, *Virgaria nigra*

CCF 2880, *Mortierella elongata* CCF 1143, *M. exigua* CCF 2860, *M. gamsii* CCF 2864, *M. humilis* CCF 2654, *M. parvispora* CCF 2867, *Mucor mucedo* CCF 2660, *M. wosnessenskii* CCF 2636, and *Rhizopus microsporus* CCF 1570.

In case of 3 strains (*Dactylaria lanosa*, *Hypoxylon serpens* and *Chalaropsis thielavioides*) the survival was slightly lower (one aliquot out of six did not form a colony after activation). This could be caused by irregular growth of these strains on perlite in cryovials, when the mycelium is not distributed evenly.

The growth rates of all strains tested were almost unaffected by the process of freezing and thawing. Although the growth of the colonies formed from the aliquots immediately after activation was mostly slightly slower (not always – quite frequently it was even faster) than that of freshly inoculated colonies, the differences disappeared in the next transfer. The period of the first occurrence of growth of the frozen cultures was different for individual cultures. Some of the strains showed signs of re-growth within 2 d, most of the strains revived within 7 d, the others in up to a maximum of 10 d after plating.

General morphology of the colonies (color, density, colony height and colony diameter) as well as micromorphological characteristics (shape of the cells or hyphae, branching of hyphae) of the recovered cultures of all strain sets showed no apparent changes in comparison with the morphology of cultures maintained by serial transfers.

Besides survival, another principal requirement for successful maintenance of fungal strains is the ability to preserve their genetic and physiological features unchanged. Routine tests carried out currently in most fungal collections to estimate the success of surviving the process of cryopreservation involve usually growth tests together with morphology assessment of fungal colonies, sometimes also certain enzyme production tests. Storage in LN is generally believed to surpass all other techniques in the preservation ability (Kocková-Kratochvílová and Hubálek 1983; Hubálek 1996; Smith 1998). Nevertheless, the papers studying these features before and after cryopreservation are rare, especially in the case of genetic characteristics. Singh *et al*. (2004) confirmed the genetic stability of 11 cryogenically preserved edible mushroom strains by comparing random-amplified polymorphic DNA (RAPD), and internal transcribed spacers (ITS)-amplified profiles.

PP has several additional advantages: one cultivation step is saved by using the cryovials directly for the cultivation of cultures; the transparency of the cryovials makes it possible to check the growth of the culture inside and prevents possible problems with insufficient inoculation and contamination. Special features of perlite (retaining and releasing water and air) affect the ice formation and thawing and this can be useful for culture protection and preservation. The mycelium on perlite grows usually continuously and its damage by punching it from the agar plate and by subsequent handling is prevented and specific mycelial structures can be preserved more easily. Moreover, the cryovials can be directly distributed without re-inoculation; the cultures on perlite can also be re-used for successive inoculations and turned out to be a good substitute for agar cultures in a long-term maintenance of fungi. Unlike conventional agar slants, cryovials with perlite are able to keep the cultures in good condition for one year or more. PP is also suitable for different sensitive fungal strains requiring special treatment (Homolka *et al*. 2001). In conclusion, we confirmed our assumption that PP can be successfully used for cryopreservation of taxonomically different groups of fungi, including yeasts, and also for fungi which failed to survive other routinely used preservation procedures.

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