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Note

Basidiomycete cultures on perlite survive successfully repeated freezing and thawing in cryovials without subculturing

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Abstract

Mycelial basidiomycete cultures on perlite in cryovials survived successfully three successive cycles of freezing, storage in liquid nitrogen (LN) and thawing without noticeable changes. This indicates that using perlite as a carrier for cryopreservation could in most cases overcome difficulties caused by interrupted supply of LN or electric power during the storage. Cultures on perlite can also be reused for successive inoculations.

Keywords: Cryopreservation; Viability; Basidiomycetes; Freezing; Thawing; Perlite; Straws; Liquid nitrogen; Laccase

Storage in liquid nitrogen (LN) has been considered the best and most widely applicable preservation technique available for filamentous fungi (Smith, 1998). It is a safe and reliable method of 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" for the preservation of fungi in LN using agar miniblocks in polypropylene straws was developed by Elliott (1976), improved by Stalpers et al. (1987) and modified by other authors (Hoffmann, 1991; Homolka et al., 2003 – straw protocol SP). The cryopreservation process includes freezing and thawing, the rate of both these processes being important (Leef and Mazur, 1978; Ryan et al., 2001). A new method of cryopreservation using perlite as a carrier of fungal mycelium (perlite protocol, PP) was developed in our laboratory originally for 5 basidiomycete strains (Homolka et al., 2001) and then successfully verified on 442 basidiomycete strains (Homolka et al., 2006). The aim of this work was testing the ability to survive three successive cycles of freezing and thawing (using PP or SP) of 50 mycelial basidiomycete cultures on perlite in cryovials. We wanted to find out if potential difficulties caused by interrupted supply of LN or electric power could be overcome in this way and also if the used cryopreservation protocol significantly influenced the survival of the cultures. The possibility of reusing the stored cryovials for successive inoculations was also tested.

In the study of repeated freezing, 50 basidiomycete strains of 49 species from different taxonomic groups, including slowly or fast growing strains with low or high laccase production, were tested using two different cryopreservation protocols – perlite protocol (PP) and straw protocol (SP). The strains were obtained from the Culture Collection of Basidiomycetes (CCBAS), Institute of Microbiology AS CR, Prague, and maintained on wort agar slants at 4 °C. Three successive cycles of freezing/thawing were performed. Six cryovials with each strain were thawed in a water bath at 37 °C, two of them were used for checking the viability and other characteristics, and the remaining four cryovials were refrozen after a short 7-day recovery at 24 °C using the respective cryopreservation protocol and stored in LN for another 2 days. In case of PP, the thawed content of the cryovials (two for each strain) was divided into three approximately equal aliquots and these were plated onto wort solid medium (wort 4° Balling, 1.5% agar Difco) in Petri dishes and incubated at 24 °C for 14 days. In case of SP, four individual straws were used instead of perlite particles. The viability was counted as percentage of surviving replicates able to form a colony after activation.

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PP was performed as follows: The fungal cultures were grown directly in sterile plastic Nunc CryoTube Vials (1.8 ml) with 200 mg of perlite (Agroperlit, agricultural grade, purchased from Keramik a.s., Prague) moistened with 1 ml of wort (4° Balling) enriched with glycerol (Sigma, final concentration 5%) as a cryoprotectant, inoculated with an agar plug (6 mm diameter) cut from an actively growing part of a colony on a Petri dish containing wort medium with glycerol (wort 4° Balling, 5% glycerol, 1.5% agar Difco) and then incubated for 14 days at 24 °C. The cryovials with perlite particles overgrown

Table 1
Viability characteristics of individual fungal species subjected to repeated freezing and thawing

Fungus	CCBAS no	PP PSS	SP PSS		Growth (mm)		ABTS test	
		After C3	After C2	After C3	Before	After C3	Before	After C3
Abortiporus biennis (Bull.:Fr.) Sing.	521	100	100	25	WD	WD	4	4
Agaricus abrubtibulbus Peck	301	100	0	0	60±5.2	55±4.8	3	3
A. arvensis Schaeff.	753	100	0	0	42±5.4	39±6.6	2	2
Agrocybe cylindracea (DC.:Fr) R.Maire	312	100	25	0	WD	WD	3	3
Antrodia heteromorpha (Fr.:Fr.) Donk.	749	100	25	0	54±4.1	48±5.7	0	0
Armillaria borealis Merx. et Korh.	833	100	75	25	49±3.6	48±4.1	4	4
A. bulbosa (Barla) Kile et Watling	678	100	25	0	51±5.5	50±5.8	4	4
A. mellea (Vahl:Fr.) Kumm.	826	100	0	0	62±7.2	62±5.6	4	4
Bjerkandera adusta (Wild.:Fr.) P. Karst	930	100	25	0	84±4.2	85±4.8	2	2
Bolbitius titubans (Bull.:Fr.) Fr.	333	100	0	0	70±8.7	67±6.2	1	1
Calvatia utriformis (Bull.:Pers.) Jaap	769	100	0	0	33±2.8	32±3.3	2	2
Clitocybe gallinacea (Scop.:Fr.) Lange	342	100	0	0	41±5.1	38±4.8	0	0
C. josserandii (Sing.) Sing.	343	100	0	0	92±8.2	91±10.1	2	2
Collybia asema (Fr.:Fr.) Kumm.	702	100	0	0	44±5.0	41±5.4	1	1
C. butyracea (Bull.:Fr.) Kumm.	347	100	0	0	33±3.7	32±3.8	3	3
Coprinus ephemerus (Bull.:Fr.) Fr.	357	100	0	0	73±6.6	71±7.1	1	1
Coriolopsis polyzona (Pers.) Ryv.	740	100	0	0	93±9.2	92±11.4	1	1
Entoloma clandestinum (Fr.:Fr.) Noordeloos	497	0 [a]	0	0	41±3.2	–	1	–
Fistulina hepatica (Schaeff.):Fr.	532	100	100	0	62±4.7	58±6.6	0	0
Fomitopsis pinicola (Sw.:Fr.) P.Karst.	536	100	50	0	71±6.2	72±7.2	0	0
Ganoderma lipsiense (Batsch) Atk.	540	100	50	0	65±5.8	63±6.1	2	2
G. lucidum (Curt.:Fr.) P. Karst.	744	100	50	0	59±4.9	61±5.3	1	1
Hericium abietis (Weir. ex Hub.) Harris.	664	100	25	0	81±7.0	80±6.4	1	1
Inonotus dryophilus (Berk.) Murr.	703	100	50	0	WD	WD	2	2
I. hispidus (Bull.:Fr.) P.Karst.	810	100	25	0	WD	WD	0	0
I. obliquus (Pers.:Fr.) Pilát	559	100	50	0	79±8.8	80±6.8	2	2
Irpex lacteus (Fr. ex Fr.) Fr.	238	100	50	0	WD	WD	1	1
Langermannia gigantea (Batsch.:Pers.) Rostk.	808	0[a]	0	0	39±3.3	–	0	–
Lycoperdon perlatum Pers.:Pers.	629	0[a]	0	0	33±4.2	–	2	–
Macrolepiota procera (Scop.:Fr.) Sing.	796	100	75	0	43±3.7	45±4.4	2	2
Onnia triquetra (Lenz) Imazeki in Ito	708	100	100	0	73±8.1	72±7.2	0	0
Oudemansiella brunneomarginata Vasil.	665	100	100	25	89±8.2	87±9.0	3	3
O. mucida (Schrad.:Fr.) Höhn.	435	100	100	0	83±6.3	82±5.8	3	3
Phaeolus schweinitzii (Fr.) Pat.	569	100	50	0	82±10.2	79±9.9	0	0
Phellinus hartigii (Allesch. et Sch.) Pat.	586	100	50	25	59±6.7	57±5.6	2	2
P. igniarius (L.:Fr.) Quél.	575	100	25	25	WD	WD	1	1
P. nigrolimitatus (Romell) Bourd. et Galz.	577	100	0	0	92±6.2	88±8.3	2	2
Pholiota adiposa (Batsch:Fr.) Kumm.	594	100	50	0	56±5.5	58±6.0	1	1
P. adiposa (Batsch:Fr.) Kumm.	683	100	50	0	70±5.9	68±6.2	2	2
Pleurotus cystidiosus O.K.Miller	466	100	50	0	60±4.4	57±6.3	4	4
P. dryinus (Pers.:Fr.) Kumm.	468	100	25	0	59±4.1	58±4.4	4	4
P. ostreatus cv.Florida (Jacq.:Fr.) Kumm.	741	100	0	0	WD	WD	4	4
P. ostreatus var. columbinus (Quél. In Bres.) Quél.	462	100	0	0	WD	WD	4	4
Polyporus ciliatus Fr.:Fr.	592	100	50	25	94±4.7	93±5.2	3	3
P. lentus Berk.	590	100	75	0	82±9.3	78±8.8	2	2
P. squamosus (Huds.):Fr.	676	100	75	0	WD	WD	3	3
P. varius (Pers.):Fr.	591	100	50	0	WD	WD	1	1
Pycnoporus sanguineus (L.:Fr.) Murr.	596	100	50	0	WD	WD	3	3
Serpula lacrymans (Wulf.:Fr.) Schroet.	110	100	100	0	WD	WD	0	0
Trametes versicolor (L.:Fr.) Pilát	612	100	100	0	WD	WD	4	4

CCBAS No = catalogue number in the CCBAS collection, SP PSS = percentage of successfully surviving agar culture plugs after storage in liquid nitrogen using straw protocol (SP), PP PSS = percentage of successfully surviving aliquots on perlite after storage in liquid nitrogen using perlite protocol (PP), C1, C2, C3 = the respective freezing/thawing, Growth = growth zone diameter (mm) on agar medium after 14-day incubation; WD = whole dish covered with mycelium, ABTS-test = laccase activity of cultures before C1 and after C3 using PP (semiquantitative four-point scale).

[a] Cultures non-viable after C2 and C3.