Folia Microbiol. **48** (2), 219–226 (2003) **http://www.biomed.cas.cz/mbu/folia/**

Viability of Basidiomycete Strains after Cryopreservation: Comparison of Two Different Freezing Protocols

L. HOMOLKA, L. LISÁ, F. NERUD

Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czechia
e-mail homolka@biomed.cas.cz

Received 12 September 2002
Revised version 23 October 2002

ABSTRACT. The viability of 250 basidiomycete strains was determined after a 2-d and then after a 2-year storage under liquid nitrogen using two different freezing protocols. Using an original agar plug protocol (OP), 162 strains (65%) of the 250 strains survived a 2-d storage and 158 strains (63 %) survived a 2-year storage in liquid nitrogen. Using a straw protocol (CP), 246 strains (98 %) of the 250 strains survived a 2-d storage and 243 strains (97 %) a 2-year storage in liquid nitrogen. In addition, other 106 strains were newly estimated using the CP protocol; 104 (98 %) of them survived successfully a 2-d storage and 101 (95 %) of them survived a 2-year storage in liquid nitrogen. The results indicate that the protocol used for cryopreservation can significantly influence strain survival. Markedly better results were obtained using the CP protocol.

Efficient mycological work requires a reliable source of cultures, *i.e.* well-defined and taxonomically determined starting material, which is ensured by its safe storage. Routine subculturing is not a very practical method of storing large numbers of fungal cultures. It is time-consuming, prone to contamination and does not prevent genetic and physiological changes during long-term and frequent subculturing. Various storage methods have been developed in order to eliminate these disadvantages. Among them, storage in liquid nitrogen has been considered to be the best and most widely applicable preservation technique available for filamentous fungi (Smith 1998). It is a safe and perspective method for long-term maintenance of most fungal species, especially those not amenable to freeze-drying. This storage technique seems to surpass all others in the ability to preserve genomic and phenotypic features and is therefore effective in most microbial biodiversity maintenance programs (Prescott and Kernkamp 1971; Heckley 1978; Smith 1982).

According to the literature and our experience, maintenance of basidiomycetes is rather difficult. Many of these fungi do not form asexual spores, their dominant form being mycelium which is rather sensitive to environmental conditions, and also mycorrhizal relationships play often a certain role. A comprehensive and detailed overview of the methods and results of cryopreservation of microorganisms, including basidiomycetes, was published by Hubálek (1996).

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 the 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 (Hwang 1968). A useful straw technique with agar miniblocks for the preservation of fungi in liquid nitrogen was developed by Elliott (1976) and improved by Stalpers *et al*. (1987). Another technique using straws in cryotubes without a cryoprotectant solution was described by Hoffmann (1991).

This paper continues the work of Chvostová *et al*. (1995), comparing the results achieved using a currently used routine cryopreservation protocol with those obtained using the original protocol. In addition, another 106 strains were newly tested, the viability after two years of storage in liquid nitrogen was retested and the ability to keep the laccase production and basic growth characteristics was evaluated.

MATERIALS AND METHODS

Organisms. The fungi used (356 strains of 173 species) are listed in Table I. They were obtained from the *Culture Collection of Basidiomycetes*, *Institute of Microbiology, Academy of Sciences of the Czech Republic* (CCBAS), Prague. The cultures were maintained by serial transfers and kept on wort agar slants at 4 °C.

Table I. Viability of individual fungal species

Species	CCBAS no.	*n*[a]	OP PSS[b]		CP PSS[b]		ABTS test[c]	
			2 days	2 years	2 days	2 years	before	after
Abortiporus biennis (BULL.:FR.) SING.	521	1	100	100	100	100	4	4
Agaricus abrubtibulbus PECK	301	1	–	–	100	100	3	4
A. arvensis SCHAEFF.:FR.	302, 303, 753	3	–	–	100	100	2	2
A. bisporus (LANGE) IMBACH	305–308	4	–	–	100	100	3	3
A. silvaticus SCHAEFF.:FR.	793	1	–	–	100	100	2	2
Agrocybe cylindracea (DC.:FR) R. MAIRE	311–319, 677	10	–	–	100	100	3	3
A. dura (BOLT.:FR.) SING.	639, 640, 832	3	–	–	100	100	3	3
A. erebia (FR.) KUHN.	641	1	–	–	100	100	3	3
A. paludosa (LANGE) KUHN. et ROMAGN.	642–644	3	–	–	100	100	3	3
Antrodia flavescens (BRES.) RYV.	652	1	100	100	100	100	2	2
A. heteromorpha (FR.:FR.) DONK.	526, 747–749	4	56	50	100	100	2	2
Armillaria borealis MERX. et KORH.	833	1	100	100	100	100	4	4
A. bulbosa (BARLA) KILE et WATLING	678	1	100	100	100	100	4	4
A. mellea (VAHL:FR.) KUMM.	324–328, 330	6	75	67	100	100	4	4
A. socialis (DC.:FR.) HERINK	331, 332	2	63	50	100	100	4	4
Aurantioporus croceus (PERS.:FR.) MURR.	522	1	100	100	100	100	2	2
Clitocybe cerussata (FR.:FR.) KUMM.	340	1	–	–	100	100	2	2
C. ditopa (FR.:FR.) GILL.	341, 773	2	–	–	100	100	1	1
C. ericetorum (BULL.) QUÉL	774	1	–	–	100	100	0	0
C. gallinacea (SCOP.:FR.) LANGE	342	1	–	–	100	100	0	0
C. josserandii (SING.) SING.	343	1	–	–	100	100	2	2
C. odora (BULL.: FR.) KUMM.	344, 835	2	–	–	100	100	2	2
C. phyllophila (FR.:FR.) KUMM.	805	1	–	–	100	100	2	2
Clitopilus passeckerianus (PILÁT) SING.	739, 738	2	–	–	100	100	3	3
C. prunulus (SCOP.:FR.) KUMM.	775	1	–	–	100	100	3	3
Collybia asema (FR.:FR.) KUMM.	702	1	–	–	100	100	1	1
C. butyracea (BULL.:FR.) KUMM.	347, 348, 754	3	–	–	100	100	3	3
C. confluens (PERS.:FR.) KUMM.	354, 801	2	–	–	100	100	3	3
C. dryophila (BULL.:FR.) KUMM.	387, 811	2	–	–	100	100	3	3
C. fusipes (BULL.:FR.) QUÉL.	349, 350	2	–	–	100	100	3	3
C. maculata (ALB. et SCHW.:FR.) KUMM.	755	1	–	–	100	100	2	2
C. marasmoides (BRITZ.) BRESINSKY et STANGL	351	1	–	–	100	100	2	2
Coniophora puteana (SCHUM.:FR.) P. KARST.	524, 525	2	75	88	100	100	–	–
Coriolopsis polyzona (PERS.) RYV.	740, 824	2	100	100	100	100	–	–
Creolophus cirrhatus (PERS.:FR.) P. KARST.	527	1	75	75	100	100	2	2
Cyathus striatus (HUDS.:PERS.) WILLD.	807	1	100	100	100	100	–	–
Daedalea quercina L.:FR.	528, 529	2	100	100	100	100	–	–
Daedaleopsis confragosa (BOLT.:FR.) SCHROET.	530, 804	2	100	100	100	100	–	–
Dichomitus squalens (P. KARST.) REID	750, 751	2	100	100	100	100	0	0
Entoloma clandestinum (FR.:FR.) NOORDELOOS	497	1	–	–	100	100	1	1
Faerberia carbonaria (ALB. et SCHW.:FR.)	668	1	–	–	100	100	3	3
Fistulina hepatica (SCHAEFF.:FR.):FR.	531–533, 809	4	94	81	100	100	–	–
Flammulina ononidis ARNOLDS	836	1	100	100	100	100	0	0
F. velutipes (CURT.:FR.) SING.	363–368	6	100	100	100	100	–	–
Fomes fomentarius (L.:FR.) FR.	534	1	0	0	100	100	–	–
Fomitopsis pinicola (SW.:FR.) P. KARST.	535–537	3	100	100	100	100	–	–
F. rosea (ALB. et SCHW.:FR.) P. KARST.	538, 539	2	100	100	100	100	–	–
Ganoderma carnosum PAT.	706	1	0	0	100	100	1	1
G. lipsiense (BATSCH) ATK.	540, 541, 745, 746	4	19	25	100	100	2	2
G. lucidum (CURT.:FR.) P. KARST.	707, 743, 744	3	0	0	100	100	1	1
G. valesiacum BOUD.	705	1	0	0	100	100	1	1
Gloeophyllum abietinum (BULL.:FR.) P. KARST.	542	1	100	100	100	100	–	–
G. sepiarium (WULF.:FR.) P. KARST.	543	1	100	100	100	100	–	–
Grifola frondosa (DICKS.:FR.) S.F. GRAY	653	1	100	100	100	100	1	1
Gymnopilus hybridus (FR.:FR.)SING.	369	1	100	100	100	100	1	1
G. junonius (FR.) ORTON	370, 650	2	50	50	100	100	2	2
G. purpuratus (COOKE et MASSEE) SING.	819	1	100	100	100	100	0	0

G. sapineus (FR.) R. MAIRE	372	1	25	25	100	100	1	1
Hapalopilus rutilans (PERS.:FR.) P. KARST.	544	1	75	75	100	100	–	–
Hericium sp.	663, 664	2	100	100	100	100	1	1
H. coralloides (SCOP.:FR.) S.F. GRAY	548–550, 661, 662, 837	6	100	100	100	100	2	2
H. flagellum (SCOP.) PERS.	551, 654	2	100	100	100	100	2	2
Hohenbuehelia petaloides (BULL.:FR.) SCHULZER	373	1	75	100	100	100	–	–
Hypholoma capnoides (FR.:FR.) KUMM.	718, 788	2	100	100	100	100	–	–
H. fasciculare (HUDS.:FR.) KUMM.	720, 812, 813	3	67	67	100	100	–	–
Hypsizygus tessulatus (BULL.:FR.) SING.	669, 722	2	75	63	100	100	–	–
Inonotus andersonii (ELL. et EVER.) ČERNÝ	557	1	75	75	100	100	0	0
I. dryadeus (PERS.:FR.) MURR.	554	1	100	100	100	100	0	0
I. dryophilus (BERK.) MURR.	703	1	100	100	100	100	2	2
I. glomeratus (PECK) MURR.	555	1	25	0	100	100	2	2
I. hispidus (BULL.:FR.) P. KARST.	810	1	100	100	100	100	0	0
I. nidus-pici PILÁT	558	1	0	0	100	100	2	2
I. nodulosus (FR.) P. KARST.	556	1	0	0	100	100	2	2
I. obliquus (PERS.:FR.) PILÁT	559,655	2	25	38	100	100	2	2
Ischnoderma benzoinum (WAHLENB.:FR.) P. KARST.	656	1	75	50	100	100	3	3
I. resinosum (FR.) P. KARST.	553	1	50	50	100	100	3	3
Kuehneromyces mutabilis (SCHAEFF.:FR.) SING. et SMITH	382–386	5	50	50	100	100	3	3
Laetiporus sulphureus (BULL.:FR.) MURR.	560–563	4	100	100	100	100	–	–
Lampteromyces japonicus (KAWAM.) SING.	388	1	0	0	100	100	0	0
Laricifomes officinalis (VILL.:FR.) KOTLABA et POUZAR	694–696	3	83	67	100	100	1	1
Lentinellus castoreus (FR.) KONR. et MAUBL.	724	1	100	100	100	100	2	2
Lentinus edodes (BERK.) SING.	389, 648, 649	3	17	8	100	100	4	4
L. lepideus (FR.:FR.) FR.	390	1	100	100	100	100	0	0
L. tigrinus (BULL.:FR.) FR.	391, 392, 826	3	100	100	100	100	3	3
Lenzites warnieri DUR. et MONT.	564	1	100	100	100	100	0	0
Lepista irina (FR.) BIGELOW	838	1	–	–	100	100	0	0
L. luscina (FR.:FR.) SING.	394, 790, 839	3	–	–	100	100	1	1
L. nebularis (BATSCH: FR.) HARMAJA	395–398, 725	5	–	–	100	100	2	2
L. nuda (BULL.:FR.) COOKE	726	1	–	–	100	100	1	1
Lyophyllum ulmarium (BULL.:FR.) KÜHN.	408	1	100	100	100	100	–	–
Meripilus giganteus (PERS.:FR.) P. KARST.	844	1	100	100	100	100	–	–
Merulius tremellosus SCHRAD.:FR.	565	1	0	0	100	100	–	–
Micromphale foetidum (SOW.:FR.) FR.	816	1	0	0	100	100	–	–
Onnia triquetra (LENZ) IMAZEKI in ITO	708	1	50	25	100	100	0	0
Oudemansiella brunneomarginata VASIL.	665	1	75	100	100	100	–	–
O. mucida (SCHRAD.:FR.) HÜHN.	425–431, 433–441, 651, 682, 732	19	46	47	100	100	0	0
O. radicata (RELH.:FR.) SING.	422, 443–447, 688, 701, 733, 767, 800	11	–	–	100	100	4	4
Panellus serotinus (SCHRAD.:FR.) KUHN.	448, 449, 734, 845	4	–	–	100	100	3	3
P. stipticus (BULL.:FR.) P. KARST.	450	1	–	–	100	100	3	3
Paxillus panuoides (FR.:FR.) FR.	451	1	100	100	100	100	0	0
Phaeolus schweinitzii (FR.) PAT.	567, 568, 569	3	100	100	100	100	0	0
Phallus impudicus L.:PERS.	632, 633, 634	3	–	–	100	100	0	0
Phanerochaete chrysosporium BURDS.	570, 571, 827–829, 854	6	100	100	100	100	0	0
Phellinus chrysoloma (FR.) DONK.	573	1	100	100	100	100	0	0
P. contiguus (PERS.:FR.) P. KARST.	758	1	100	100	100	100	0	0
P. hartigii (ALLESCH. et SCH.) PAT.	586	1	75	50	100	100	2	2
P. igniarius (L.:FR.) QUÉL.	574–576	3	75	75	100	100	1	1
P. laevigatus (P. KARST.) BOURD. et GALZ.	657	1	75	75	100	100	3	3
P. nigrolimitatus (ROMELL) BOURD. et GALZ.	577	1	100	100	100	100	2	2
P. pini (BROT.:FR.) AMES	578	1	100	100	100	100	1	1
P. robustus (P. KARST.) BOURD. et GALZ.	587	1	100	100	100	100	3	3
P. torulosus (PERS.) BOURD. et GALZ.	759	1	100	100	100	100	3	3
P. vorax (HARKNESS) ČERNÝ	735	1	100	100	100	100	0	0
Phlebiopsis gigantea (FR.:FR.) JÜLICH	572	1	100	100	100	100	0	0

continued

Table I – *continued*

Pholiota adiposa (BATSCH:FR.) KUMM.	452, 453, 594, 683, 736	5	100	100	100	100	2	2
P. aurivella (BATSCH:FR.) KUMM.	454	2	100	100	100	100	2	2
	846		100	100	100	100	0	0
P. destruens (BROND.) GILL.	670	1	50	25	100	100	2	2
P. flavida (SCHAEFF.:FR.) SING.	455	1	–	–	100	100	3	3
P. lenta (PERS.:FR.) SING.	456, 780	2	50	50	100	100	3	3
P. spumosa (FR.) SING.	848	1	0	0	50	25	1	1
P. squarrosa (MÜLL.:FR.) KUMM.	457, 458	2	50	50	100	100	3	3
Piptoporus betulinus (BULL.:FR.) P. KARST.	579–585	7	93	86	100	100	–	–
Pleurocybella porrigens (PERS.:FR.) SING.	460, 768	2	50	50	100	100	3	3
Pleurotus calyptratus (LINDBL. in FR.) SACC.	461	1	0	0	100	100	4	4
P. citrinopileatus SING.	691	1	0	0	100	100	4	4
P. cornucopiae (PAUL.) ROLLAND	463–465, 675	4	38	25	100	100	4	4
P. cystidiosus O.K. MILLER	466	1	100	100	100	100	4	4
P. dryinus (PERS.:FR.) KUMM.	467–470	4	94	100	100	100	4	4
P. eryngii (DC.:FR.) QUÉL.	471	1	100	100	100	100	4	4
P. ostreatus (JACQ.:FR.) KUMM.	472–476	5	45	50	100	100	4	4
P. ostreatus cv. *Florida* (JACQ.:FR.) KUMM.	477, 741	2	0	0	100	100	4	4
P. ostreatus var. *columbinus* (QUÉL. in BRES.) QUÉL.	462	1	0	0	75	50	4	1
P. pulmonarius (FR.) QUÉL.	478–481, 684	5	10	15	100	100	4	4
P. sajor-caju (FR.) SING.	666	1	50	50	100	100	4	4
P. salmoneostramineus VASIL.	692	1	100	100	100	100	4	4
Pluteus petasatus (FR.) GILL.	483, 485, 757	3	33	33	67	67	0	0
Polyporus badius (S.F. GRAY) SCHW.	818	1	–	–	100	100	0	0
P. brumalis (PERS.:FR.) FR.	588, 589	2	100	100	100	100	3	3
P. ciliatus FR.:FR.	592, 799	2	100	100	100	100	3	3
P. lentus BERK.	590	1	0	0	50	25	2	1
P. squamosus (HUDS.:FR.):FR.	676	2	50	0	75	50	2	1
	850		50	75	100	100	3	3
P. varius (PERS.) FR.	591	1	0	0	75	50	1	1
Psathyrella candolleana (FR.:FR.) R. MAIRE	486	1	–	–	75	100	1	1
P. piluliformis (BULL.:FR.) ORTON	487	1	–	–	50	50	1	1
Psilocybe cubensis (EARLE) SING.	488	1	–	–	75	50	1	1
P. cyanescens WAKEF. emend. KRIEGLST.	489, 490, 672, 714, 820	5	–	–	100	100	3	3
P. semilanceata (FR.) KUMM.	492, 494	3	–	–	75	75	3	3
	493		–	–	50	50	2	1
Pycnoporus cinnabarinus (JACQ.:FR.) P. KARST.	595, 787	2	100	100	100	100	3	3
P. sanguineus (L.:FR.) MURR.	596	1	100	100	100	100	3	3
Pyrofomes demidoffii (LV.) KOTLABA et Pouzar	597	1	–	–	75	50	1	1
Rhodocybe gemina (FR.) KUYPER et NOORDELOOS	496	1	–	–	100	100	2	2
Rhodotus palmatus (BULL.:FR.) R. MAIRE	498	1	100	100	100	100	3	3
Rigidoporus populinus (SCHUM.:FR.) POUZAR	598	1	100	100	100	100	0	0
Sarcodontia setosa (PERS.) DONK	599	1	0	0	100	100	3	3
Schizophyllum commune FR.:FR.	600–603, 752, 851–853	8	100	100	100	100	2	2
Scleroderma citrinum PERS.	637	1	–	–	50	50	1	1
S. verrucosum (BULL.) PERS.	830	1	–	–	75	50	1	1
Serpula lacrymans (WULF.:FR.) SCHROET.	110	1	–	–	100	100	–	–
Sparassis crispa WULF.:FR.	606, 607	2	0	0	100	100	–	–
S. nemecii PILÁT et VESELÝ	658	1	0	0	100	100	–	–
Stereum hirsutum (WILLD.:FR.) FR.	608	1	75	100	100	100	3	3
Trametes gibbosa (PERS.:FR.) FR.	609, 806	2	100	100	100	100	4	4
T. hirsuta (WULF.:FR.) PILÁT	610, 742	2	100	100	100	100	4	4
T. suaveolens FR.	611	1	100	100	100	100	2	2
T. versicolor (L.:FR.) PILÁT	612, 613, 614	3	100	100	100	100	4	4
Trichaptum abietinum (PERS.:FR.) RYV.	552	1	100	100	100	100	3	3
Tubaria furfuracea (PERS.: FR.) GILL.	519	1	–	–	100	100	1	1
Tyromyces caesius (SCHRAD.:FR.) MURR.	673	1	–	–	100	100	0	0
T. lacteus (FR.) MURR.	616	1	–	–	100	100	0	0
T. stipticus (PERS.:FR.) KOTLABA et POUZAR	615, 690	1	–	–	100	100	2	2

Vascellum pratense (PERS.:PERS.) KREISEL	698	1	–	–	75	75	0	0
Xerula longipes (BULL.) R. MAIRE	520	1	–	–	100	100	3	3
Xylobolus frustulatus (PERS.:FR.) BOIDIN	704	1	–	–	100	100	0	0

[a]Number of tested strains.
[b]Percentage of successfully surviving (PSS) agar culture plugs after 2-d or 2-year storage in liquid nitrogen using original (OP) and current (CP) protocol.
[c]Laccase activity (semiquantitative four-point scale, *see Materials and Methods*) of cultures before and after cryopreservation using CP.

Culture preparation and freezing/thawing protocols. Original cryopreservation protocol (OP) using agar plugs was performed using a modified combined procedure according to Butterfield *et al*. (1978) and Smith and Ward (1987). Four wort agar plugs (diameter 3 mm) cut from an actively growing part of colony were transferred into sterile screw-capped polyethylene cryovials (3.6 mL) filled with 2 mL of protective solution (glycerol–water 1 : 9, *V*/*V*). After precooling to 7 °C, the cultures were cooled to –35 °C for 45–60 min and then immersed directly in liquid nitrogen.

Our current routine cryopreservation protocol (CP), which is slightly modified from Hoffmann's protocol (Hoffmann 1991), is the following: Strains are grown on Petri dishes on MEYA medium (in %, *V*/*V*: malt extract 2, yeast extract 0.2, *Difco* agar 1.5, glycerol 5) at 24 °C. Sterile, thin (diameter 1 mm) plastic straws, open at both ends, are used to punch the mycelium with agar from the colony. This is repeated until the straws are filled with agar miniplugs containing the mycelium. These filled straws are then transferred into sterile polypropylene cryovials, tightly sealed with screw stoppers provided with silicone gaskets and frozen in a programmable freezer *Planer* R202 to –70 °C at a freezing rate of 1 K/min. Frozen cultures were put into liquid nitrogen.

Viability control. Thawing (reactivation of cultures) was common for both tested protocols. After a 2-d or 2-year storage in liquid nitrogen, the cryovials were transferred to a water bath (37 °C) until the ice completely melted. Prior to opening, the surface of the cryovials was disinfected with ethanol. After thawing, the agar plugs or straws were incubated on wort or MEYA agar plates at 25 °C for up to 14 d, all in quadruplicate. Strains exhibiting 75–100 % survival of separate plugs or straws were considered viable. The strains of the same species were involved in the evaluation only when their viability reached 75–100 %.

Laccase test. For checking the enzyme production stability a semiquantitative color laccase test according to Niku-Paavola *et al*. (1990) was used. Extracellular laccase activity was tested on 2-week-old colonies before and after storing in liquid nitrogen. Before staining, the mycelia were removed from the agar surface by scraping with a scalpel. 2,2′-Azinodi(3-ethylbenzothiazoline-6-sulfonic acid; ABTS) was used to detect the laccase activity by pouring the ABTS solution on the solid media in dishes. A fresh staining solution (5 g/L distilled H_2O) was prepared before use. The intensity of blue-green color was estimated after 10 min using a four-point scale (0–4).

RESULTS AND DISCUSSION

The viability of 250 strains (representing 120 species of 80 genera) of wood-inhabiting basidiomycetes has been retested and viability of 106 other strains (representing 54 species of 23 genera) newly tested after storage in liquid nitrogen with glycerol as cryoprotective.

The recovery of the tested strains and their laccase activity are summarized in Table I. The principal criteria for a successful recovery of fungal strains were the ability to retain original growth, macro- and micromorphological characteristics, and unchanged production of laccase.

The growth rates of all strains were virtually unaffected by the process of freezing and thawing. The activity of laccase before cryopreservation and after the revival usually did not differ substantially. There were only two exceptions: in *Polyporus squamosus* CCBAS 676 and *Psilocybe semilanceolata* CCBAS 493 it decreased slightly from 2 to 1 point. General morphology of the colonies (color, density, mycelium height and colony diameter) and micromorphological characteristics of the recovered cultures showed no apparent changes in comparison to the morphology of cultures maintained by serial transfers, which is in agreement with Stoytchev *et al.* (1998). The period of the first occurrence of growth of the frozen cultures was different in both protocols. Some of the strains showed signs of regrowth within 2 d, most of the strains revived within 7 d, the others up to maximum of 14 d after plating. Even after further two transfers on fresh solid media the results were almost the same (*data not shown*). No contamination was detected.

Table II illustrates that using the OP protocol, only of ⅔ strains survived a 2-d or a 2-year storage in liquid nitrogen, respectively. Using a straw CP protocol, their survival increased up to 95–97 %. Other

106 strains were newly tested using the CP protocol, their survival being also high (95–97 %). The cryopreservation CP protocol used can then significantly influence survival; considerably better results were achieved using this protocol.

Table II. Viability of *Basidiomycetes* preserved using OP and CP protocols after storage in liquid nitrogen for 2 days or 2 years

Protocol	OP		CP				Total	
Set	original		original		new			
Storage	2 days	2 years	2 days	2 years	2 days	2 years	2 days	2 years
Genera								
Tested	58		58		23		80	
Viable[a]	47	45	57	57	23	21	79	77
%	81	78	98	98	100	91	99	96
Species								
Tested	120		120		53		173	
Viable[a]	78	73	117	115	51	49	168	164
%	65	61	97	96	96	92	97	95
Strains								
Tested	250		250		106		356	
Viable[a]	162	158	246	243	104	101	350	344
%	65	63	98	97	98	95	98	97

[a]Survival of agar culture plugs or straws 75–100 %.

In case of OP, attempts to use this procedure for cryopreservation of the genera *Fomes*, *Ganoderma*, *Lampteromyces*, *Merulius*, *Micromphale*, *Sarcodontia*, *Sparassis* and some species of *Inonotus*, *Pholiota*, *Pleurotus* and *Polyporus* failed completely (Table I). In the species of the last 4 mentioned genera the success rate varied surprisingly between 0 and 100 %. An unexpected response to cryopreservation using OP was observed in strains of the same species. Nineteen strains of *O. mucida*, 5 strains of *K. mutabilis* and 5 strains of *P. ostreatus* exhibited a success rate 0–100 %. In 6 strains of *A. mellea* and 4 strains of *A. heteromorpha* the success rate varied between 25 and 100 %. This different response to the freezing-thawing process is therefore an intrinsic feature of the respective strain.

Quite different results were obtained using CP, where no such "unsuccessful" genera or species occurred and only few intraspecies divergences were found. This is evidently caused by the differences in the protocols used. The improved cryopreservation CP procedure differs from the original OP protocol particularly in the way of freezing and protection of mycelial plugs. Two types of cooling rates, either slow (controlled) or rapid (uncontrolled), have been employed in the cryopreservation process (Jong 1978; Alexander *et al*. 1980).

The majority of fungi are not sensitive to rapid freezing if the warming is rapid, but better recovery was reported for controlled freezing (Jong and Davis 1986). The freezing process in CP was controlled – a freezing rate of 1 K/min was used, the cultures frozen to at least –70 °C were immersed in liquid nitrogen, and 4 straws, each containing several miniplugs, were placed inside a single cryotube. In the case of OP, 4 larger agar plugs were placed directly into the protective glycerol solution in cryovials and no glycerol was added to the agar medium used for precultivation. Our strains (in which we observed problems using OP) may be particularly sensitive to the cooling rate. We believe that by variation of the initial cooling rate survival of the strains can be improved (Challen and Elliott 1986; Morris *et al*. 1988) and our recent results support this view. The viability of some fungi that are usually difficult to freeze might be achieved using precooling in the refrigerator at 4 °C (Smith 1982). Our results show that even a slow and controlled precooling is not suitable for some of the fungi tested. Nevertheless, the process of recovery using the OP protocol can be complicated in some fungal strains due to the separation of the mycelial mat from the discs in the cryopreservation solution (Homolka *et al*. 2001).

To determine the real influence of individual factors on the survival would require many demanding experiments, where the results could be uncertain. From our fragmentary ambiguous results concerning only several samples (*e.g.*, uncontrolled freezing of straws, freezing of large agar plugs enriched with glycerol, *etc.*) it seems that the crucial factor is the controlled freezing.

Another factor of great importance is a suitable cryoprotectant. Many compounds have been used either alone or in combination (Withers 1980). Glycerol and dimethyl sulfoxide proved to be most successful (Jong and Atkins 1985). We found that glycerol is a good protectant for almost all strains. Otherwise, Stoychev *et al*. (1998) showed that glycerol is comparable with Me_2SO.

Data on the production of metabolites or other physiological features of the preserved strains are relatively rare (Hubálek 1996). With *O. mucida*, the ability to produce the antibiotic mucidin was practically unaffected by cryopreservation (Homolka 1976). With some white-rot basidiomycetes, we found no negative effect of cryopreservation or the cryoprotective used on the production of ligninolytic enzymes (Stoychev *et al*. 1998). Retaining of laccase production was used in our study as an auxiliary criterion for successful survival of the strains tested. Except for the two above strains, all others kept their laccase production unchanged. Nevertheless, the semiquantitative nature of the test must be taken in account.

The survival of fungi tested is in a good agreement with the results published earlier (Hwang 1968; Hwang *et al*. 1976; Challen and Elliot 1986; Jong and Davis 1986; Smith and Ward 1987) and compare favorably with those reported by major world collections.

Despite of relatively high efficiency of the cryopreservation procedures currently used for fungal cultures (especially basidiomycetes) these are far from being flawless and in particular versatile. A need for an alternative method of cryopreservation arose after several partially or completely unsuccessful attempts to preserve some of our basidiomycete strains or their specific properties by our current routinely used procedure using straws in cryovials and by a standard cryotechnique using agar discs in polypropylene vials with a cryoprotectant solution. Therefore we carried out a preliminary study of the possible use of perlite as carrier of mycelial cultures of a few problematic basidiomycete strains and the results were encouraging (Homolka *et al*. 2001).

This work was supported by *Institutional Research Concept* no. AV 0Z 502 0903.

REFERENCES

ALEXANDER M., DAGGETT P.-M., GHERNA R., JONG S.C., SIMIONE F., HATT H.: *American Type Culture Collection Methods I. Laboratory Manual on Preservation Freezing and Freeze Drying*. American Type Culture Collection, Rockville (USA) 1980.

BUTTERFIELD W., JONG S.C., ALEXANDER M.T.: Polypropylene vials for preserving fungi in liquid nitrogen. *Mycologia* **70**, 1122–1124 (1978).

CHALLEN M.P., ELLIOTT T.J.: Polypropylene straw ampoules for the storage of microorganisms in liquid nitrogen. *J.Microbiol.Meth*. **5**, 11–23 (1986).

CHVOSTOVÁ V., NERUD F., HOMOLKA L.: Viability of wood-inhabiting basidiomycetes following cryogenic preservation. *Folia Microbiol.* **40**, 193–197(1995).

ELLIOTT T.J.: Alternative ampoule for storing fungal cultures in liquid nitrogen. *Trans.Brit.Mycol.Soc.* **67**, 545–546 (1976).

HECKLEY R.J.: Preservation of microorganisms. *Adv.Appl.Microbiol*. **24**, 1–53 (1978).

HOFFMANN P.: Cryopreservation of fungi. *World J.Microbiol.Biotechnol.* **7**, 92–94 (1991).

HOMOLKA L.: On the problem of maintenance and cultivation of higher fungi. *Folia Microbiol.* **21**, 189–190 (1976).

HOMOLKA L., LISÁ L., EICHLEROVÁ I., NERUD F.: Cryopreservation of some basidiomycete strains using perlite. *J.Microbiol.Meth.* **47**, 307–313 (2001).

HUBÁLEK Z.: *Cryopreservation of Microorganisms*. Academia, Prague 1996.

HWANG S.-W.: Investigation of ultra-low temperature for fungal cultures – I. An evaluation of liquid nitrogen storage for preservation of selected fungal cultures. *Mycologia* **60**, 613–621 (1968).

HWANG S.-W., KWOLEK W.F., HAYNES W.C.: Investigation of ultra-low temperature for fungal cultures – III. Viability and growth rate of mycelial cultures following cryogenic storage. *Mycologia* **68**, 377–387 (1976).

JONG S.C.: Conservation of the cultures, pp. 119–135 in *The Biology and Cultivation of Edible Mushrooms* (S.S.Chang, W.A.Hay, Eds). Academic Press, New York 1978.

JONG S.C., ATKINS W.B.: Conservation, collection and distribution of cultures, pp. 153–194 in *Fungi Pathogenic for Humans and Animals. Part B, Pathogenicity and Detection II* (H.D.H. Howard, Ed.). Marcel Dekker, New York 1985.

JONG S.C., DAVIS E.E.: Germplasm preservation of edible fungi in culture through cryogenic storage, pp. 213–225 in *Proc. Internat. Symp. Scientific and Technical Aspects of Cultivating Edible Fungi.* The Penna State University, University Park (USA) 1986.

MORRIS G.J., SMITH D., COULSON G.E.: A comparative of the changes in the morphology of hyphae during freezing and viability upon thawing for twenty species of fungi. *J.Gen.Microbiol*. **134**, 2897–2906 (1988).

NIKU-PAAVOLA M.L., RAASKA L., ITAVAARA M.: Detection of white-rot fungi by a non-toxic stain. *Mycol.Res.* **94**, 27–31 (1990).

PRESCOTT J.M., KERNKAMP M.F.: Genetic stability of *Puccinia graminis* f.sp. *tritici* in cryogenic storage. *Plant Dis.Rep.* **55**, 695–696 (1971).

SMITH D.: Liquid nitrogen storage of fungi. *Trans.Brit.Mycol.Soc.* **79**, 415–421 (1982).

SMITH D.: The use of cryopreservation in the *ex-situ* conservation of fungi. *Cryo-Letters* **19**, 79–90 (1998).

SMITH D., WARD S.M.: *Notes on the Preservation of Fungi*. CAB International Mycological Institute, Kew (UK) 1987.

STALPERS J.A., DE HOOG A., VLUG I.J.: Improvement of the straw technique for the preservation of fungi in liquid nitrogen. *Mycologia* **79**, 82–89 (1987).

STOYCHEV I., HOMOLKA L., NERUD F., LISÁ L.: Activities of ligninolytic enzymes in some white-rot basidiomycete strains after recovering from cryopreservation in liquid nitrogen. *Antonie van Leeuwenhoek* **73**, 211–214 (1998).

WITHERS L.A.: Low temperature storage of plant tissue. *Adv.Biochem.Eng.* **18**, 102–150 (1980).