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Preservation of hyphal-forming brown- and white-rot wood-inhabiting basidiomycetes

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ABSTRACT

Lyophilization is an excellent technique for the long-term preservation of hyphal-forming brown- and white-rot wood-inhabiting basidiomycotina. However, vegetative mycelial isolates are not lyophilizable. In this study, 10 brown-rot and 10 white-rot basidiomycetous non-sporulating, non-chlamydosporulating, and non-bubillerferous basidiomycetes fungi were tested for viability after lyophilization. Optimization of the lyophilization involved (i) using mycelium agar plugs and mycelial fragments, (ii) improving physiological growth conditions, age of cultures, and growth medium components, (iii) adding to and adjusting the cold hardening period, initial-freezing rates, and dehydration and rehydration, and (iv) standardizing lyoprotectant type and concentration. All fungal growth on solid agar medium or in broth survived lyophilization. No noticeable changes in viability or morphological and biochemical characteristics occurred. Some mycelial isolates exhibited heavy filamentous mycelial growth and/or grew faster after lyophilization.

Key Words: basidiomycetes, bulbils, chlamydospore, conidium, cryopreservation, liquid nitrogen, lyophilization, skimmed milk, trehalose, lyoprotectant.

INTRODUCTION

Mycelial isolates of brown- and white-rot, wood-inhabiting basidiomycotina usually grow well as vegetative hyphae at optimal growth conditions on artificial media. It is well known that successive transfer onto artificial media is not only labor intensive but also increases the possibility of genetic drift. Maintaining the mycelial isolates is, therefore, of great importance. Nonchlamydosporulating, and nonbubillerferous basidiomytes (Croan et al., 1999) and nonsporulating basidiomycetes that exist only as filamentous or vegetative hyphal-forming fungi do not survive lyophilization and are therefore reported to be non-lyophilizable fungi (Rybnikar 1985, 1995; Smith, 1984; Tan et al., 1994). Most lyophilizable or non-lyophilizable fungi survive cryopreservation using liquid nitrogen (Kurtzman, 1980, 1986; Maekawa et al 1990; Tan et al., 1997). However, liquid nitrogen is expensive and requires constant resupply. The requisite labor is not always available.

Hyphal-forming basidiomycetes were lyophilized through removal of water under vacuum from a frozen state by sublimation of ice. This procedure allows biological time to be stopped so that cultures can be stored for long-term preservation. Lyophilized cultures in ampoules can easily be stored in a small space, require no maintenance, and can be shipped without special

requirements. Thus, lyophilization represents an excellent alternative technique for long-term preservation and is also a useful alternative to cryopreservation using liquid nitrogen.
Here we describe the complete process protocol developed to lyophilize the vegetative mycelium of hyphal-forming, brown- and white-rot, wood-inhabiting basidiomycetes with no change in their original characteristics.

MATERIALS AND METHODS

Fungi tested. —The dikaryotic isolates of brown- and white-rot wood-inhabiting basidiomycetes obtained from the Center for Forest Mycology Research at Forest Products Laboratory (USDA Forest Service, Madison, WI) were selected for long-term preservation. The CFMR collection number of each isolate is listed in Table I.

Media. –The mycelium isolates were inoculated to malt extract agar (MEA; 1.5% (w/v) malt extract; Bacto, Difco, Detroit, Michigan, 2% (w/v) agar (Bacto, Difco), and two growth media, PIMGT [Potato Infusion (Atlas, 1993) with 10 g malt extract, 2 g glucose, and 20g trehalose, with or without 20 g Bacto-agar per 1000 mL] or MPT (15 g malt extract, 5 g peptone, and 20 g trehalose, with or without 20 g Bacto-agar per 1000 mL). Other agar media were prepared by adding 0.5% (w/v) gallic acid (GAA) (Mallinckrodt Baker, Inc., St. Louis, Missouri) or tannic acid (TAA) (Mallinckrodt Baker, Inc.) to MEA, using the procedure of Davidson et al (1938). Tyrosine (0.2%, w/v; Tyr, Sigma) was added to 1.5% Bacto-agar (Boidin 1958) with 0.5% malt extract (Nakasone 1990). All 90-mm-diam agar plates and 30-mm-diam MPGT agar plates were inoculated at the center with a 6-mm-diam plug taken from the margin of a young, actively growing colony. The plug was inoculated at the center of each plate and incubated at 24 C in the dark until measurable growth occurred.

Lyophilization. – Mycelium was lyophilized by means of two different methods, mycelium/agar plug for the fast-growing isolates and fragments from mycelial mats for the slow-growing isolates. For the mycelium/agar plug method, 5 agar plugs with mycelium of a stationary growth colony on MPGT agar or PIMGT agar were transferred into cotton-plugged 2-mL sterile constricted glass ampoules. The mycelium from the plugs in the ampoules was allowed to grow around the entire surface of the agar plugs for 1 to 3 wk at 24^{O} C. Approximately 0.5 to 0.6 mL of a mixture of 10% (w/v) skimmed milk (Difco) with 10% (w/v) D (+) trehalose (Sigma, St. Louis, Missouri) as the lyoprotectant was added to the ampoules.

For the mycelial fragment method, a mycelium/agar plug of a young, actively growing margin of the colony on MEA was inoculated into 10 mL MPGT or PIMGT broth in a 125-mL Erlenmeyer flask until the mycelial mat covered the surface of the medium. The mycelial mat was washed with sterile 0.005% Tween 20 in distilled water, torn into 5-6 pieces, and then centrifuged. Approximately 0.2 to 0.3 g wet weight of the mycelial pellet was aseptically transferred into cotton-plugged 2-mL sterile constricted ampoules. The mycelial fragments in the ampoules were added to approximately 0.3 to 0.4 mL of the mixture of 10% (w/v) skimmed milk (Difco) with 10% (w/v) D (+) trehalose and allowed to grow further around the surface of the mixture for 2 to 3 wk at 24^{O}C. An additional 0.2 mL of the mixture of 10% (w/v) skimmed milk (Difco) with

10% (w/v) D (+) trehalose was added to the ampoules with filamentous growth to make a final volume of 0.5 to 0.6 mL. The ampoules were then transferred to the refrigerator (4 C) overnight to permit a cold hardening period (Smith, 1984). Using liquid nitrogen as the coolant, the ampoules were slowly frozen (Hwang and Howells, 1968; Kurtzman, 1980; Smith, 1984; Smith and Onions, 1983, 1994, Tan et al. 1995) in a microcomputer-programmed freezer (Cryomed Model 1010 programmable cooler, subsidiary of Stremikon, Mt. Clemens, MI) to about -45^{O}C at a rate of -1^{O}C /min, then frozen to -90^{O}C at a rate of -10^{O}C/min. The frozen samples were dehydrated under vacuum in an ethanol-dry-ice bath. After complete dehydration, the ampoules were then sealed under vacuum and stored in the refrigerator. The sealed ampoules were opened after a period ranging from a few days to a few weeks. Sterile distilled water was added to the lyophilized cultures and left for approximately10 to 30 min to permit absorption of the water. These reconstituted lyophilized specimens, whether mycelium/agar plugs or mycelial fragments, were then inoculated on MEA plates in order to determine their viability. The results were compared to those obtained within a period of a few days to a few weeks. The growth rate was determined by measuring the diameters of the fungal colonies on MEA every 2 to 3 days during 2 weeks incubation at 24^{O}C.

The sealed ampoules were opened after a period of a few days to a few weeks. Sterile distilled water was added to rehydrate the lyophilized cultures and left for approximately 30 to 45 min to permit water absorption. These reconstituted lyophilized specimens (mycelium/agar plugs) were then inoculated on MEA plates to determine their viability. The growth rate of viable cultures was determined by measuring the diameter of the fungal colonies on MEA every 2 to 3 d during 2 wk incubation at 24^{O}C.

Gallic acid, tannic acid, and tyrosine agar medium reactions. –Specific strain characteristics were compared before and after preservation to ascertain the stability of the isolates. The viability of the isolates before and after preservation was ascertained by testing the ability of vegetative hyphae to regrow on MEA plates. Growth characteristics and agar medium reactions on gallic acid, tannic acid, and tyrosine were also compared before and after preservation.

A mycelium/agar plug (6-mm-diam) of a young, actively growing margin of the colony on MEA plates was inoculated at the center of the gallic acid, tannic acid, and tyrosine agar plates. The growth rate and color reactions were recorded at 1- and 2-wk intervals after incubation in the dark at 24^{O}C. The rate of radial growth was also compared to that of the growth on the 1.5% MEA plates. The reaction of the diffusion zone to the fungal colony of the tropical mycelial isolates was rated according to the method described by Davidson et al. (1938) with minor revisions (Table II). Two additional reactions were also recorded: (i) diffusion zone light yellow, formed under the inoculum plug, and (ii) diffusion zone black, formed under the inoculum plug on the tyrosine agar medium when no growth takes place.

RESULTS

Tables I and II list the 20 isolates which were selected for study: 10 brown-rot basidiomycetes (designated B) and 10 white-rot basidiomycetes (designated W). The collection (CN) and working (SC) numbers of the isolates are listed in Table I. Mycelium/agar plug grown on PIMGTA or MPTA (growth medium with 2% trehalose) were used for the fast-growing

basidiomycetes (B8, B10, W1, W2, W3, W5, W6, W7, W8, and W10). Mycelia fragments grown in 10 mL PIMGT or MPT broth in a 125-mL Erlenmeyer flask were used for slow-growing basidiomycetes (B1 through 9, W3, and W9). Mycelium/agar plugs and mycelium fragments harvested during the stationary phase survived lyophilization when the mycelium/agar plugs and mycelia fragments were frozen by slow cooling in a mixture of skimmed milk with trehalose after cold storage at 4^{o}C. The isolates exhibited filamentous growth and/or grew faster compared to nonlyophilized isolates. Mycelium on agar plugs grown on MEA or mycelium mats grown on MEB (malt extract broth) under the same conditions did not survive lyophilization.
Growth of about half the mycelial isolates was apparently stimulated after lyophilization, compared to the growth of nonlyophilized cultures. These isolates exhibited filamentous growth (Table I, Fils) and/or grew faster (Table I, FG) on MEA plates immediately after lyophilization compared to nonlyophilized isolates. The other surviving isolates grew slowly after lyophilization, but normal growth characteristics were usually obtained after the second transfer to MEA plates. Lyophilization affected the rate of growth, but none of the isolates demonstrated any change in their morphological and anatomical characteristics.

The nonlyophilized isolates were compared to the isolates that survived the preservation process. The agar medium reaction of the nonlyophilized isolates to gallic acid, tannic acid, and tyrosine were basically the same as that of mycelial isolates that survived after lyophilization (Table II).

DISCUSSION

Brown- and white-rot, hyphal-forming basidiomycetes generally grow well as vegetative hyphae under optimal growth conditions of temperature, aeration, and humidity with artificial media as. The hyphal-forming basidiomycetes tend not to survive under unfavorable conditions of lyophilization unlike thick-walled bulbils-, chlamydospore-, or conidium-forming basidiomycetes. In our study, the mycelial isolates were sufficient to initiate good fungal growth on a growth medium containing 2% trehalose and 0.2% glucose. Bacteria can use exogenous trehalose as the sole source of carbon and energy as well as synthesize enormous amounts of disaccharide (Arguelles, 2000). *Saccharomyces cerevisiae* was reported to have at least two different trehalose transport systems, (i) supporting cellular growth as a source of carbon and (ii) transporting the exogenous trehalose through the plasma membrane into intracellular trehalose (Malluta et al., 2000). The mycelial isolates of *Pleurotus ostreatus* (SC 23; Croan, unpublished data) that were grown in PIMG broth containing trehalose accumulated intracellular trehalose up to 18% trehalose per mycelial dry weight. The isolates also exhibited filamentous growth and/or grew faster after lyophilization compared to nonlyophilized isolates. Mycelium/agar plugs grown on malt-extract agar did not survive lyophilization, possibly because there was no intracellular trehalose accumulation to protect the mycelial isolates during the unfavorable conditions of the lyophilization process. When the trehalose-grown mycelium/agar plugs were directly frozen by immersion in a dry ice–ethanol bath, they did not survive lyophilization, possibly because intracellular solutes leaked during the transition from active life to a rapidly frozen state. This suggests that a slower cooling rate followed by the lyophilization process may be necessary to assure the viability of these tropical basidiomycetes (Croan, 2000).

Trehalose-grown isolates apparently absorbed additional trehalose intracellularly during the process of lyophilization in the presence of trehalose-containing lyoprotectant. Viability in the presence of trehalose as the lyoprotectant by *lyophilizable* bacteria was reported to improve considerably (Leslie et al 1995). Trehalose might have accumulated rapidly and thus protected the bacterial cells during freezing and rehydration.

All trehalose-grown mycelium/agar plugs or mycelial fragments of isolates that exhibited the filamentous growth on/in growth medium survived lyophilization in presence of the trehalose as the lyoprotectant. These trehalose-grown isolates appeared to be protected against cell damage in the presence of trehalose.

The fact that some isolates exhibited filamentous growth and/or grew faster on MEA plates immediately after lyophilization may have been due to the survival of the more vital parts of mycelia through lyophilization. The cultures of B1, B6, B8, B10, W1, W2, W4, W5, W6, and W10 grew faster with filamentous growth on MEA after lyophilization. The other surviving isolates grew slowly after lyophilization, but normal growth rates were usually obtained after the second transfer to MEA plates. Irrespective of whether growth rates were affected by lyophilization, none of the isolates demonstrated any change in morphological and anatomical characteristics or biochemical properties (agar medium reactions of tannic acid, gallic acid, and tyrosine), suggesting that no major genetic changes occurred.

In summary, this technique demonstrates an effective method for preserving brown-rot and white-rot, wood-inhabiting basidiomycetes and possibly many other basidiomycetes as well.

LITERATURE CITED

Boidin J. 1958. Essai biotaxonomique sur les hydnes resupuines et les corticies. Rev Mycol (Paris) Memoire hors-serie 6:39–42.

Croan SC, Burdsall HH Jr, Rentmeester RM. 1999. Preservation of tropical wood-inhabiting Basidiomycotina. Mycologia 91: 908-916.

Croan SC. 2000. Lyophilization of hypha-forming tropical wood-inhabiting Basidiomycotina. Mycologia 912: 8110-817.

Davidson RW, Campbell WA, Blaisdell DJ. 1938. Differentiation of wood-decaying fungi by their reactions on gallic or tannic acid medium. J Agric Res 57:683–695.

Hwang S, Howells A. 1968. Investigation of ultra-low temperature for fungal cultures. II. Cryoprotection afforded by glycerol and dimethyl sulfoxide to 8 selected fungal cultures. Mycologia 60:622–626.

Kurtzman CP. 1980. Preservation of fungi by lyophiliization and liquid nitrogen freezing. *In: preservation of microorganisms by freezing and freeze-drying.* Annual Meeting of Society for Industrial Microbiology. August 9–10, 1980. Flagstaff: Northern Arizona University. p. 4-12.

Leslie SB, Israeli E, Lighthart B, Crowe JH, Crowe LM. 1995. Trehaolose and sucrose protect both membranes and proteins in intact bacteria during drying. Appl Environ Microb 61:3592-3597.

Maekawa N, Fukuda M, Arita I, Komatsu M. 1990. Effects of liquid-nitrogen cryopreservation on stock cultures of three cultivated basidiomycetous fungi. Rept Tottori Mycol Inst 28: 227–232.
Malluta EF, Decker P, and Stambuk BU. 2000. The Kluyver effect for trehalose in Saccharomyces cerevisiae. J. Basic Microbiol. 40 (3), 1999-205.

Nakasone KK. 1990. Cultural studies and identification of wood-inhabiting Corticiaceae and selected Hymenomycetes from North America. Mycol Mem 15:4–15.

Rybnikar A. 1985. Storage of lyophilized dermatophyte cultures. ACTA VET BRNO 54:79–83.

Rybnikar A. 1995. Long-term maintenance of lyophilized fungal cultures of the genera *Epidermophyton*, *Microsporium*, *Paecilomyces* and *Trichophyton*. Mycoses 38:145–147.

Smith D. 1984. Maintenance of fungi. Pp. 83–107. *In: Maintenance of microorganisms.* Eds., B. E. Kirsop and J. S. Snell. Academic Press, London, United Kingdom.

Smith D, and Onions AHS. 1983. A comparison of some preservation techniques for fungi. Trans Br Mycol Soc 81:535–540.

Smith D, aand Onions AHS. 1994. *The preservation and maintenance of living fungi.* IMI Technical Handbooks, no. 2, 122, 2d edition. CAB International. Wallingford Oxon, United Kingdom.

Tan CS. 1997. Preservation of fungi. Cryptogamie Mycologist 18:157–163.

Tan CS, Vlug IJA, Stalpers JA, Van Ingen CW. 1994. Microscopical observations on the influence of the cooling rate during freeze-drying of conidia. Mycologia 86:281–289.

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Table I. Survival of mycelial isolates of brown- and white-rot basidiomycetes after lyophilization

Fungus[a]	Coll_no.	SC no.	Mycelium/agar plugs and Mycelia fragments
*Antrodia radiculosa**	FP-90848	B1	3/5[b], 2+, FG
*Antrodia serialis**	FP-104443	B2	4/5+
*Antrodia sinuosa***	FP-105386	B3	5/5, 2+
*Coniophora puteana***	FP-102011	B4	5/5, 2+
*Fomitopsis pinicola***	FP-105877-R	B5	5/5, 2+
*Gloeophyllum sepiarium***	Mad-604	B6	5/5, 2-3+, FG
*Gloeophyllum trabeum***	Mad-617	B7	5/5, 2+
*Laetiporus sulphureus***	Sh-27-R	B8	5/5, 2+, FG
*Neolentinus lepideus***	Mad-534	B9	2-4/5+
*Positia placenta**	Mad-698	B10	5/5, 3+, Fils
*Bjerkandera adusta**	L-15359	W1	5/5, 3-4+, FG
*Ceriporiopsis subvermispora**	CZ-3	W2	5/5 3+ or 4+, Fils & FG
*Ganoderma applanatum***	RLG-6298	W3	3-4/5 + or ++
*Irpex lacteus**	HHB-7328	W4	5/5,3-4+, Fils & FG
*Phlebia brevispora**	HHB-7030	W5	5/5, 3-4+, Fils & FG
*Phlebia tremellosa**	Fp-101416	W6	5/5, 2+, FG
*Pycnoporus cinnabarinus**	FP-100498	W7	5/5, 2+
*Schizophyllum commune**	Mad-619	W8	5/5, 2+
*Stereum complicatum***	FP-101692	W9	5/5, 2+
*Trametes versicolor**	Mad-697	W10	5/5, 2-3+, Fils

[a] Single asterisk (*) by the name of the fungus denotes trehalose-grown mycelium/agar plugs of isolates that survived after lyophilization;
**denotes mycelial fragments of isolates grown in MPGT broth that survived after lyophilization.
[b]Fractions indicate number of mycelium/agar plugs; numerator is number of plugs that survived after lyophilization; denomerator is total number of plugs. Whole numbers indicate quality of growth: 1+, very poor, 3–5 wk; 2+, slow growth, within 2–3 wk; 3+, moderate growth, 1–2 wk; 4+, excellent growth, within 3 d to 1 wk. Fils, filamentous growth; FG, fast growth.

Table II— Gallic acid, tannic acid, and tyrosine media reactions of isolates[a]

Fungus	SC no.	Coll_no..	MEA GR (mm)	GAA GR (mm)		**TAA** GR (mm)		**Tyr A** GR (mm)	
					rxn		rxn		rxn
Antrodia radiculosa	B1	FP-90848	35	32	-	0	-	18	-
			75	65	-	0	-	55	-
Antrodia serialis	B2	FP-104443	28	14	-	tr	-	14	-/+*
			63	31	-	21	-	45	-/+*
Antrodia sinuosa	B3	FP-105386	38	16	-	0	-	24	-
			65	36	-	0	-	52	-
Coniophora puteana	B4	FP-102011	34	20	-	0	-	14	-
			62	50	+-	0	-	25	-
Fomitopsis pinicola	B5	FP-105877-R	34	20	-	15	-	32	-
			73	45	-	26	-	45	-
Gloeophyllum sepiarium	B6	Mad-604	35	12	-	0	-	20	-
			70	25	-	0	-	50	-
Gloeophyllum trabeum	B7	Mad-617	40	12	-	0	-	22	-
			78	32	-	0	-	45	-
Laetiporus sulphureus	B8	Sh-27-R	46	44	-	28	-	14	-
			90+	90	-	90	-	60	-
Neolentinus lepideus	B9	Mad-534	46	11	-	0	-	26	-
			78	25	-	0	-	50	-
Positia placenta	B10	Mad-698	41	tr	-	35	-	20	-
			90	90	-	18	-	45	-
Bjerkandera adusta	W1	FP-101236	78, dc	0	-	0	-	46	-
			90+, dc	tr	-+	0	-+	90	-
Ceriporiopsis subvermispora	W2	CZ-3	90+, dc	55	++	tr	++	68	+-
			90+, dc	70	3-4+	35	2-3+	90	+
Ganoderma applanatum	W3	RLG-6298	21	0	-	0	++	17	-
			43	0	0	12	+++	36	-
Irpex lacteus	W4	HHB-7328	66, dc	0	+-	0	-	56	+-
			90+, dc	tr	+-	0	+-	90	++
Phlebia brevispora	W5	HHB-7030	70+, dc	10	+++	0	-	tr	+*
			90+, dc	40	3-4+	0	-	90	++*
Phlebia tremellosa	W6	Fp-101416	74, dc	0	+++	tr	+-	72	+
			90+, dc	0	4+	37	++	90	++
Pycnoporus cinnabarinus	W7	FP-100498	90+, dc	0	+++	tr	+++	90	+
			90+, dc	0	+++	37	+++	90	+
Schizophyllum commune	W8	Mad-619	46, dc	12	++	12	++	41	-
			90, dc	17	+++	27	+++	90	+
Stereum subtomentosum	W9	HHB-7544/	21	0	-	tr	-	12	-
			35-49	18	-	0	-	59	-
Trametes versicolor	W10	Mad-697	62	tr	+++	0	++	53	-
			90+, dc	0	+++	37	+++	90	-

[a]GAA, TAA, and TyrA reactions: Radial growth rate and color reactions were recorded after 1 and 2 wk incubation.
First row of data indicates measurement after 1 wk incubation; second row, measurement after 2 wk.
GR designates growth radius; dc, decolorized MEA; rxn, reaction; 90+, designates maximum growth.

– no change
+- diffusion zone light yellow, formed under inoculum plug
+ diffusion zone light to dark brown, formed under inoculum plug at center of colony or under inoculum plug when no growth takes place
++ diffusion zone light to dark brown, formed under most of colony or under inoculum plug in the event that no growth takes place
+++ diffusion zone light to dark brown, extending short distance beyond margin of colony or inoculum plug when no growth takes place
++++diffusion zone dark brown, extending considerably beyond margin of colony or inoculum plug when no growth takes place
* diffusion zone black instead of light or dark brown