Mycologia, 91(5), 1999, pp. 908-916.
© 1999 by The Mycological Society of America, Lawrence, KS 66044-8897

Preservation of tropical wood-inhabiting basidiomycetes

Suki C. Croan[1]
Harold H. Burdsall, Jr.
Rita M. Rentmeester

Center for Forest Mycology Research, U.S. Department of Agriculture, Forest Service, Forest Products Laboratory, Madison, Wisconsin 53705-2398[2]

Abstract: The objective of this study was to develop a method to preserve tropical wood-inhabiting basidiomycetes in a viable state for an extended period. Storage in water, lyophilization, and cryopreservation methods of preserving tropical wood-inhabiting basiomycotina were compared. When tropical mycelial isolates were stored in sterile distilled water, 57% survived at 4 C for 2.5 mo; 92% of the isolates survived at 15 C for 10 mo. Bulbil-, clamydospore-, and conidium-forming fungi survived in cold water storage and maintained excellent viability for a decade. Vegetative hypha-, bulbil-, clamydospore-, and conidium-forming fungi survived cryopreservation using liquid nitrogen vapor. Only basidiomycetes that formed bulbils, chlamydospores, or conidia survived lyophilization. We conclude that the best method for long-term preservation of tropical fungi is cryopreservation by means of storage in liquid nitrogen vapor.

Key Words: basidiome, bulbil, chlamydospore, conidium, cryopreservation, lyophilization, lyoprotectant, water storage

INTRODUCTION

Fungi are diverse groups of heterotrophs, some of which produce secondary metabolites that may be used for biotechnological purposes. Wood-inhabiting basidiomycetes can be a valuable resource for new pharmaceuticals, food production, bioremediation of toxic chemical spills (Kirk et al 1992b, Lamar et al 1994), biopulping of paper, and other industrial uses (Akhtar et al 1993, Kirk et al 1992a). These fungi maintain the productivity and sustainability of natural resources and contribute to a cleaner environment.

Mycelial isolates of tropical wood-inhabiting basidiomycetes generally grow well as vegetative hyphae at optimal growth temperatures on artificial media. Most tropical isolates that have reached maximum growth on malt extract agar (MEA) plates have not maintained viability after 2 mo at 4 C or after 6 mo at 8 to 10 C. The cold temperature has been found to be detrimental to these isolates, unlike isolates from wood-inhabiting basidiomycetes from temperate climates (Croan unpubl). Maintenance of tropical wood-inhabiting basidiomycetes has not been effective. In addition, maintaining tropical isolates on artificial agar slants or plates is labor intensive and increases genetic drift in the cultures. Tropical isolates are therefore problematic with respect to both short- and long-term preservation. Dependable methods are needed for yielding viable, morphologically and physiologically unchanged microorganisms after long-term preservation. Definitive data are lacking regarding the survival of most taxa of tropical wood-inhabiting basidiomycetes when subjected to various means of long-term preservation.

The techniques for fungal preservation range from those methods that incorporate continuous growth through methods that reduce growth and metabolism to methods that arrest growth and metabolism. The most common methods for fungal preservation focus on reducing growth and metabolism. Storage in soil, mineral oil, silica gel, or distilled water is used for relatively short-term preservation of fungi, which may be adequate and offer sufficient genetic stability in some cases. However, these storage methods are labor intensive and time consuming.

Storage of conidium-, spore-, or hyphae-forming fungi on agar medium under sterile distilled water is an easy, rapid, and inexpensive method of preservation that does not result in physiological changes for many years (Boesewinkel 1976, Burdsall and Dorworth 1994, Marx and Daniel 1976, Richter and Bruhn 1989, Smith 1993). In one study, 151 different species of wood-inhabiting basidiomycetes were shown to have maintained their viability for 7 yr when stored in distilled water at 4 to 5 C (Burdsall and Dorworth 1994).

Accepted for publication April 23, 1999.
[1] Email: scroan/fpl@fs.fed.us
[2] The Forest Products Laboratory is maintained in cooperation with the University of Wisconsin. This article was written and prepared by U.S. Government employees on official time, and it is therefore in the public domain and not subject to copyright. The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

TABLE I. System for rating GAA, TAA, and TyrA medium reactions and enzyme activities

Agar medium[a] or enzyme	rxn[b]	Diffusion zone color	Diffusion zone reaction
GAA, TAA, TyrA	−	No change	No change
	±	Light yellow	Formed under inoculum plug
	+	Light to dark brown	Formed under inoculum plug at center of colony or under inoculum plug in absence of growth
	++	Light to dark brown	Formed under most of colony or under inoculum plug in absence of growth
	+++	Light to dark brown	Extends a short distance beyond margin of colony or inoculum plug in absence of growth
	≥++++	Dark brown	Extends considerably beyond margin of colony or inoculum plug in absence of growth
	*	Black	
Catalase			
	+		Slow reaction; weak positive (weak evolution of bubbling)
	++		Slow reaction; strong positive (continuous evolution of bubbling)
	+++		Immediate reaction; strong positive
laccase	−	No change	
	±	Faint reddish brown	Formed under and around colony
	+	Light reddish brown	Sometimes randomly formed under and around colony
	++	Light to dark reddish brown	Formed under and around most of colony
	+++	Reddish brown	Extends short distance beyond margin of colony
Peroxidase	−	No change	
	±	Faint reddish brown	Formed under and around colony
	+	Light to dark reddish brown	Formed around and under most of colony
	++	Light to dark reddish brown	Extends short distance or slightly beyond margin of colony
	**	No change with H_2O_2	

[a] GAA is gallic acid agar; TAA, tannic acid agar; TyrA, tyrosine agar.
[b] Reporting symbols used in TABLE IV.

Lyophilization and cryopreservation by liquid nitrogen, which arrest growth and stop the metabolic process, also represent effective techniques for long-term preservation. Conidium- or spore-forming fungi preserve their original characteristics after lyophilization (Antheunisse 1973, Ellis and Roberson 1968, Haskins 1957, Haynes et al 1955, Rybnikar 1985, 1995, Schipper and Bekker-Holtman 1976, Smith 1984, Smith and Onions 1983, Tan et al 1991, 1994, Wickerham and Flickinger 1946). A wide range of nonlyophilizable fungi (filamentous or hypha-forming fungi) and lyophilizable fungi (spore- or conidium-forming fungi) easily survive liquid nitrogen storage (Daily and Higgins 1973, Hwang 1960, 1966, Hwang et al 1976, Kurtzman 1980, 1986, Maekawa et al 1990, Onions 1983, Tan 1997).

The objective of this study was to develop reliable methods to preserve secondary mycelial isolates of tropical wood-inhabiting basidiomycetes with no change in their original characteristics. We compared preservation in sterile distilled water and storage at 4 or 15 C, lyophilization and storage at 4 C and room temperature, and cryopreservation in liquid nitrogen. Fungal growth rates, morphological characteristics, and selected enzyme activities were compared before and after preservation.

TABLE II. Optimum temperature and growth temperature range of secondary mycelial isolates of tropical wood-inhabiting *basidiomycotina*

Fungus	Accession number	Growth rate at optimum temp (mm/d)	Temperature range (C)	Optimum temperature (C)
Amethecium sp.	14 653	12.6–12.9	16–44	32
Bourdotia sp.	14 718	5.5–7.7	12–32	24
Bourdotia sp.	14 726	6.7–7.1	12–32	24–28
Ceriporiopsis mucida	14 648	3.1–3.3	20–36	24–28
Cerocorticium molle	14 802	4.5–8.8	12–36	24–28
Dendrothele sp.	14 799	5.9–8.2	12–32	24
Epithele sp.	14 686	9.7–9.7	8–40	32
Formitella supina	14 618	5.7–7.6	12–44	32
Grammothele lineata	14 659	14.6–15	12–44	32
Hyphoderma ayresii	14 644	12.6–12.8	16–32	32
Hyphoderma ayresii	14 806	14.6–15	12–44	28–32
Hyphoderma ayresii	14 682	10.7–11.1	12–44	32
Hyphodontia lanata	14 792	4.0–4.1	12–32	24
Hyphodontia sp.	14 658	3.7–5.5	16–32	24–28
Peniophora s.s.	14 703	12.6–12.9	8–44	28–32
Peniophora s.s.	14 734	10.1–14.1	20–44	28–32
Phanerochaete sp.	14 720	5.5–7.1	16–32	24
Phellinus gilvus	14 607	3.3–4.7	16–36	28
Phlebia "queletii"	14 728	5.5–6.7	12–44	24–28
Phlebia "queletii" complex	14 670	7.1–8	12–32	28
Poria sp.	14 616	12.9–13.1	8–44	28–32
Porogramme albocinctus	14 612	12.6–13.1	16–44	32
Radulomyces sp.	14 791	6.1–6.4	12–24	24
Resinicium bicolor	14 642	8.3–10.2	16–44	32
Resinicium furfurellum	14 760	3.3–3.7	12–32	20–24
Rigidosporus sp.	14 608	5.8–8	24–32	28
Schizopora radula	14 641	3.0–4.4	16–32	24
Schizopora flavipora	14 605	5.5–10	16–44	28–32
Scytinostroma sp.	14 646	4.5–5.0	12–32	24
Scytinostroma sp.	14 748	5.5–6.2	8–32	24–28
Subulicystidium longisporum	14 673	TR[a]	NT[b]	NT[b]
Trechispora rigida	14 758	2.5–3.3	16–32	24
Tyromyces galactinus	14 609	4.0–41	20–32	32
Undetermined species	14 620	14.8–15	12–44	28
Undetermined species	14 633	4.1–4.1	8–32	24

[a] Trace of growth.
[b] Not tested.

MATERIALS AND METHODS

Fungi.—Tropical basidiomes were collected from woody substrates in wet to moist forests in Puerto Rico at the El Verde Field Station and Sabanna Research Station in the Caribbean National Forest, Luquillo Mountains, and Guajataca Commonwealth Forest. The specimens were collected during a 2-wk period in June 1996 and were later identified by K. K. Nakasone.

Thirty-five isolates of tropical wood-inhabiting basidiomycetes, isolated from the context tissue or spore prints of basidiomes, were selected for long-term preservation **(TABLE II).** The CFMR accession number of each isolate is listed in TABLE II. The following basidiomycetes were used for positive controls: bulbilliferous *Sistotrema biggsiae* Hallenb. GB-NH-782, chlamydospore-forming *Hyphochnicium analogum* (Bourd. & Galz.) J. Erikss. ME-800, and conidium-forming *Phanerochaete chrysosporium* Burdsall in Burds. & Eslyn [BKM-F-1767, ATCC 24725].

Media.-The fungi were plated on 1.5% (w/v) MEA (Bacto, Difco, Detroit, Michigan) 90-mm-diameter plates by placing a 6-mm-diameter mycelium/agar plug at the center of the plate and incubating at 24 C in the dark until measurable growth had occurred. Plates were incubated at 11 different temperatures, from 4 to 44 C, at 4 C intervals (4, 8, 12, 16, 20, 24, 28, 32, 36, 40, and 44 C). Colony diameter were measured every 2-3 d for 2 wk.

TABLE III. Growth rate of isolates after water storage at 4 and 15 C and after cryopreservation

Fungus	Growth rate (mm/d)[a]			
	Prior to storage or cryopreservation	Water storage at 4 C	Water storage at 15 C	Cryopreservation
Amethecium sp.	7.9–9.8	NG	7.0–10	4.7–8.5
Bourdotia sp.	5.4–7.7	NG	3.0–6.5	4.0–7.0
Bourdotia sp.	6.7–7.8	0–TR*	2.9–6.5	4.0–6.7
Ceriporiopsis mucida	2.5–3.3	NG	TR–2.6*	2.1–2.2
Cerocorticium molle	4.1–6.8	2.3–2.9	3.4–5.9	6.0–6.2
Dendrothele sp.	4.5–6.2	4.0–4.3	TR–3.8*	4.7–5.1
Epithele sp.	7.6–12.7	NG	11–12.4	10.3–11
Formitella supina	3.0–5.7	0–TR*	3.3–5.0	4.0–5.0
Grammothele lineata	10–10.7	10.5–11.4	11.3–12.4	13–13.3
Hyphoderma ayresii	4.6–5.3	NG	6.3–9.4	2.9–6.6
Hyphoderma ayresii	9.3–10	7.3–9.8	6.7–9.9	4.4–8.9
Hyphoderma ayresii	7.6–10.7	3.5–3.7	2.8–4.3	5.7–9.5
Hyphodontia lunata	2.6–4.1	NG	TR–2.6*	3.0–3.1
Hyphodontia sp.	3.7–5.5	0–TR*	0–TR*	4.0–4.4
Peniophora s.s.	10.1–12.3	12.1–12.7	11.6–11.9	11.6–12.0
Peniophora s.s.	10.1–11.1	8.9–9.4	11.6–11.9	9.6–11.0
Phanerochaete sp.	5.5–6.3	0–TR*	TR*	3.4–5.1
Phlelbia "queletii"	5.5–8.5	3.6–3.8	5.7–10.2	4.7–5.1
Phlebia "queletii" complex	6.0–9.4	5.7–6.0	7.3–11.9	8.6–9.0
Phellinus gilvus	3.1–5.3	2.1–3.3	TR*	3.5–4.5
Poria sp.	10.1–11.7	12.1–12.2	11.1–12.4	10.1–10.3
Porogramme albocinctus	11.4–12.3	12–12.6	7.0–12.4	11.3–12.1
Radulomyces sp.	4.3–6.1	NG	3.8–4.1	3.3–3.6
Resinicium bicolor	7.1–9.5	NG	4.7–8.2	6.7–8.6
Resinicium furfurellum	2.3–3.7	TR*	0–TR*	1.4–2.2
Rigidosporus sp.	3.3–3.6	NG	NG	3.6–4.0
Schiropora flavipora	4.3–8.0	3.8–6.2	4.0–4.2	4.4–6.7
Schizopora radula	3.0–4.4	NG	2.9–3.7	2.9–3.7
Scytinostroma sp.	4.5–5.0	NG	3.7–5.2	2.9–6.6
Scytinostroma sp.	5.5–6.2	NG	6.7–8.8	3.4–6.4
Subulicystidium ongisporum	TR	NG	NG	0–TR
Trechispora rigida	TR–2.0	NG	NG	1.5–1.7
Tyromyces galactinus	3.2–6.4	NG	TR–6.5	4.0–4.2
Undetermined species	10.1–11.1	10.6–12	8.0–8.9	9.8–11.7
Undetermined species	4.1–4.3	2.3–2.8	3.3–6.1	4.7–5.6

[a] NG = no growth; TR = trace of growth; 0–TR = NG wk 1 and TR wk 2; * = prolonged lag growth.

Preservation methods.—For distilled water storage, 4-5 mycelium/agar plugs of a young, actively growing margin of the colony were transferred to sterile 2-mL polyproplene cryovials; the cryovials were filled with sterile distilled water and stored at 4 or 15 C. Positive control organisms had been stored in distilled water at 4 C for 10-12 yr. After 2.5 mo at 4 C and 10 mo at 15 C, the radial growth rate was determined by inoculating the preserved agar plugs onto MEA plates and incubating in the dark at 24 C for 2 wk or until measurable growth occurred (Burdsall and Dorworth 1994).

Mycelial fragments were prepared for lyophilization by gently scraping young hyphae on MEA plates with 0.005% Tween 20 and then centrifuging. Approximately 0.5 mL of a mixture of mycelial pellets and 10% bovine (ox) serum (CS-1337, Colorado Serum Co., Denver) or 10% (w/v) skimmed milk (Difco) and 5% (w/v) myo-inositol (Sigma, St. Louis, Missouri) as the lyoprotectant were added to a 2-mL sterile constricted ampoule. The samples were frozen in an acetone dry-ice bath and then lyophilized. The ampoules were sealed under vacuum and stored at room temperature or at 4 C in the refrigerator. Immediately after lyophilization and again after 1 yr of storage, ampoules were opened and their contents were reconstituted with sterile distilled water for 10 min to permit the contents to rehydrate. The reconstituted lyophilized material was streaked onto MEA plates and viability was determined. The surviving fragments (both fragments that had been stored at room temperature and those stored in the refrigerator) were tested again to determine the more effective temperature for storage.

TABLE IV. Media and enzymatic reactions of isolates[a,b]

Fungus	MEA GR (mm)	GAA GR (mm)	GAA rxn	TAA GR (mm)	TAA rxn	TyrA GR (mm)	TyrA rxn	Catalase	Laccase	Peroxidase
Amethecium sp.	75	0	−	0	−	63	−	+/++	−	−
	90+	0	−	0	−	90+	−			
Bourdotia sp.	72	0	−	0	−	66	−	++	−	±
	90+	0	−	0	−	90+	−			
Boudotia sp.	64	0	−	0	−	46	−	+	−	±
	90+	0	++	0	++	90+	+			
Ceriporiopsis mucida	12	0	++	10	+	10	−	+	−	−
	29	0	+++	23	+++	18	+			
Cerocorticium molle	32	0	+	0	+	18	−	+	−	−
	72	0	++	35	+++	35	±			
Dendrothele sp.	42	0	+	25	++	21	−	++	−	−
	72	0	++	43	++	45	±			
Epithele sp.	88	0	+	33	++	88	−	++	±	**
	90+	0	+++	62	++	90+	+			
Formitella supina	34	0	+	0	+	25	−	+	+	**
	60	0	+	12	+++	45	±			
Grammothele lineata	50	0	+	23	+++	71	−	+/++	+	++
	90+	0	++++	62	++++	90+	++			
Hyphoderma ayresii	32	0	−	0	−	20	−	+/++	±	−
	64	0	−	0	+	90+	++			
Hyphoderma ayresii	58	0	−	0	−	20	−	+	−	−
	90+	0	±	0	−	90+	+			
Hyphoderma ayresii	78	0	−	0	−	70	+	+	±	−
	90+	0	+	0	+	90+	+			
Hyphodontia lanata	25	0	+	0	−	0	−	+	−	−
	44	0	++	0	±	0	+*			
Hyphodontia sp.	34	0−		0−		0	−	+	−	−
	67	0	−	0	−	0	−			
Peniophora s.s.	70	34	+++	43	++	75	+	+	−	−
	90+	63	++++	76	+++	90+	+++			
Peniophora s.s.	86	14	+++	56	++	45	−	+/++	±	**
	90+	20	++++	70	++	86	−			
Phanerochaete sp.	46	21	−	0	−	42	−	++	−	−
	78	31	−	0	−	90+	−			
Phellinus gilvus	43	12	+	25	+	0	+	+	−	±
	70	14	++++	45	++	0	++			
Phlebia "queletii"	69	0	+	0	−	70	+	+	+	**
	90+	0	++	0	+	90+	++			
Phlebia "queletii" complex	86	0	+	0	+	70	+	+	+	**
	90+	0	++	0	+	90+	+			

TABLE IV. Continued

Fungus	MEA GR (mm)	GAA GR (mm)	GAA rxn	TAA GR (mm)	TAA rxn	TyrA GR (mm)	TyrA rxn	Catalase	Laccase	Peroxidase
Poria sp.	68	0	+	6	+	61	−	++	−	−
	90+	0	++	10	++	90+	+			
Porogramme albocinctus	70	0	+	26	+++	71	−	++	+++	**
	90+	0	++++	41	++++	90+	++			
Radulomyces sp.	34	0	+	0	+	9	−	+++	+	**
	62	0	++	0	+	12	±			
Resinicium bicolor	77	0	+	0	−	50	−	+/++	−	−
	90+	0	++	0	±	90+	−			
Resinicium furfurellum	19	0	+	0	+	0	−	+	+	**
	34	0	++	0	++	12	−			
Rigidoporus sp.	20	0	±	0	−	22	−	+++/++	+++/+	**
	70	0	+	0	−	55	+			
Schizopora radula	45	0	+	28	+	29	±	+	+/±	**
	76	0	3+	49	++	47	+			
Schiropora flavipora	54	0	+	0	−	0	−	+	−	−
	90+	0	++	0	−	0	−			
Scytinostroma sp.	45	0	+	27	++	38	−	+	+/±	**
	74	0	+++	51	+++	60	+			
Scytinostroma sp.	60	0	+	34	+	40	−	+/++	+	**
	90+	9	+++	60	++	60	+			
Subulicystidium longisporium	0	0	−	0	−	0	−	NT	NT	NT
	8	0	−	0	−	0	−			
Trechispora rigida	8	0	+	0	±	8	−	+	−	−
	13	0	+	0	±	14	+			
Tyromyces galactinus	20	0	+	0	+	10	−	+	++/+	**
	90+	0	++	0	+++	90+	++			
Unkown sp.	64	0	+	0	+	61	−	+	++/+	**
	90+	0	+++	0	++	90+	++			
Unkown sp.	25	0	+	13	+++	15	−	+	±	−
	65	0	++	34	+++	36	++			
Hypochnicium	53	0	−	0	−	49	−	+	−	−
	90+	17	−	0	−	90+	−			
Phanerocheate chrysosoporium	90+	11	−	0	−	90+	−	++	−	−
	90+	90+	−	0	−	90+	−			
Sistotrema biggsiae	15	0	−	0	−	0	−	+++/++	−	−
	36	0	−	0	−	0	−			

[a] GAA, TAA, and TyrA reactions—Radial growth rate and color reactions were recorded after 1 and 2 wk following incubation. First row of data indicates measurement after 1 wk incubation; second row of data indicates measurement after 2 wk incubation. 90+ designates maximum growth.

[b] See TABLE I for rating system.

For cryopreservation, four to five mycelium/agar plugs of 1- to 4-wk-old cultures were transferred to sterile 2-mL polyproplene cryovials after their growth had been well-established on MEA. The cryovials were first filled with 10% glycerol as a cryoprotectant, then transferred to a refrigerator for 4 h for cold hardening (Smith 1984). Using liquid nitrogen as the coolant, the capped cryovials were frozen slowly (Hwang and Howells 1968, Smith 1984, Tan et al 1995) in a computer-programmed freezer (Cryomed Model 1010 programmable cooler, subsidiary of Stremikon, Mt. Clemens, Michigan) to about -45 C at a rate of -1 C / min then frozen to -90 C at a rate of -10 C/min, and finally stored in liquid nitrogen vapor.

After 10 mo storage, the cryovials were thawed by immersion in a reciprocal shaker at 37 C for about 30 to 45 s or until the last traces of ice had disappeared. The mycelium/agar plugs in the cryovials were inoculated at the center of the MEA plates and the isolates were tested for survival after 10 mo storage in liquid nitrogen vapor. The growth rate (mm/d) was determined by measuring the colony diameter on MEA every 2 to 3 d during 2 wk of incubation at 24 C.

Media and enzymatic 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 measured by the ability of bulbils, chlamydospores, and conidia to germinate or vegetative hyphae to regrow on MEA plates. Growth characteristics, reactions on gallic acid, tannic acid, and tyrosine agar media, guaiacol reaction with or without hydrogen peroxide for presence of laccase and peroxidase, and catalase activities were also compared before and after preservation. Agar media were prepared by adding 0.5% (w/v) gallic acid (GAA) (Mallinckrodt Baker, Inc., St. Louis, Missouri), 0.5% (w/v) tannic acid (TAA) (Mallinckrodt Baker, Inc.), or 0.2% (w/v) tyrosine (TyrA, Sigma) to MEA. Gallic and tannic acids were prepared following the procedure of Davidson et al (1938). Tyrosine was added to 1.5% bacto-agar (Boidin 1958) with 0.5% malt extract (Nakasone 1990).

A mycelium/agar plug (6-mm-diameter) of a young, actively growing margin of the colony was inoculated onto MEA plates. Radial growth rate was compared before and after preservation. Growth rate and color reactions were recorded at 1- and 2-wk intervals after incubation in the dark at 24 C. The rate of radial growth was also compared to that of growth on 1.5% MEA plates. The GAA, TAA, and TyrA reactions to the fungal colony of the isolates were rated as described by Davidson et al (1938) with minor revision (TABLE I). Two additional reactions were also recorded: diffusion zone light yellow, formed under the inoculum plug (±) and diffusion zone black, formed under the inoculum plug when *no* growth takes place (*) **(TABLE I).**

A modified test method (Garraway et al 1989, Harkin and Obst 1973)–a rapid plate assay procedure (Croan unpubl)–was used to determine the presence of laccase- and peroxidase-producing isolates. First, 1- to 2-wk-old mycelial growth on MEA plates was flooded with 25 mM guaiacol (Aldrich, Milwaukee, Wisconsin) in 5 mM $KHPO_4$ (Sigma), pH 6, to determine the presence of laccase. If no reaction occurred, the plate was then flooded again with 2 mM H_2O_2 (Mallinckrodt) to determine the presence of peroxidase. laccase and peroxidase activities were rated as shown in **TABLE I.**

To determine the presence of catalase activity, approximately 0.5 mL of 3% hydrogen peroxide was placed on a 1- to 3-wk-old colony on a MEA plate to determine the presence of catalase-producing isolates. The evolution of continuous bubbling was examined immediately and then again after a few min for positive reaction (Smibert and Krieg 1981). Catalase activity was rated as shown in **TABLE I.**

RESULTS

The tropical mycelial isolates generally either did not grow or grew poorly at lower temperatures (i.e., temperatures below 4 to 20 C, depending on fungus) and had a much higher optimum growth temperature than that of isolates from temperate areas (TABLE II). For nearly half the mycelial isolates, the optimum temperature for growth was 32 C. Four isolates grew at 20 C or higher, but *Rigidosporus* sp. grew only at 24 C or higher. Most isolates were able to grow, albeit slowly, at 12 to 16 C, and five isolates were able to grow at 8 C. After isolates had achieved maximum growth on MEA plates, they were stored for 6 mo at 8-10 C. When these isolates were transferred to new MEA plates at 24 C, less than 50% of them survived. The surviving isolates grew very slowly.

When isolates were stored in sterile distilled water at 4 C for 2.5 mo, 43% (15 of 35 isolates) failed to survive when transferred to MEA plates; however, when stored in sterile water at 15 C for 10 mo, 92% (32 of 35 isolates) survived when transferred to MEA plates **(TABLE III).** Without water, only 42% (14 of 34) vegetative hyphae of these isolates were able to grow when they were incubated at 16 C or higher (TABLE II). After storage in water at 4 C, 5 of 20 surviving fungi showed a prolonged lag period with only minimal growth **(TABLE III).** After storage in water at 15 C, 7 of 32 fungi also exhibited a prolonged lag period and had to be incubated for a longer period. Normal growth was usually obtained after the second or occasionally the third transfer to MEA plates.

None of the hyphal-forming tropical mycelial isolates survived lyophilization. The chlamydospore-, conidium-, and bulbil-forming basidiomycetes survived lyophilization. The chlamydospore- and conidium-forming basidiomycetes (*Hypochnicium analogum* and *Phanerocheate chrysosporium*, respectively) survived lyophilization with either 10% bovine serum or 10% (w/v) skimmed milk and 5% (w/v) inositol. The bulbilliferous basidiomycete, *Sistotrema biggsiae,* survived only in a mixture of skimmed milk and inositol. The lyophilized control cultures remained viable and

stable after lyophilization and during storage from 2 wk to 1 yr at room temperature or in the refrigerator.

All mycelial isolates survived cryopreservation by slow prefreezing and storage at ultralow temperature in liquid nitrogen vapor. Approximately 10% of the cryopreserved mycelia showed either slightly higher or slightly lower growth rates compared to that of isolates prior to preservation. The mycelial isolates showed little or no difference in viability, radial growth **(TABLES III, IV),** or media and enzymatic reactions **(TABLE IV).** Cryopreservation did not change the anatomical or morphological characteristics of these isolates. All mycelial isolates proved to be viable for 10 mo after cryopreservation **(TABLE III).**

The original isolates cultured from the context tissue or spore prints of tropical basidiocarps were compared to isolates that survived the preservation process. Reactions of original isolates to gallic acid, tannic acid, and tyrosine media and catalase, laccase, and peroxidase activities were basically the same as those of mycelial isolates that survived after preservation **(TABLE IV).**

DISCUSSION

Mycelial isolates generally grow well as vegetative hyphae under optimal growth conditions of temperature, aeration, humidity, and artificial media. In this study, storage at cold temperatures was found to be detrimental to tropical mycelial isolates because they were in the form of vegetative hyphae and did not have the thick-walled bulbils, chlamydospores, and conidia that are usually necessary for survival under severe conditions (cold, desiccation, exogenous chemical influence, physical pressure, and abrasion) and maintenance for extended periods.

Water storage at 15 C was found to maintain viability of the tropical wood-inhabiting basidiomycetes better than did cold (4 C) water storage or incubation without water at 16 C or higher. All positive control organisms survived after 10 to 12 yr in water storage at 4 C. Thus, the results indicate that bulbil-, clamydospore-, and conidium-forming fungi can survive cold water storage for an extended period. At both 15 C and 4 C water storage, about 25% of the surviving fungi showed a prolonged lag period with only minimal growth. Normal growth was usually obtained after the second or occasionally the third transfer to MEA plates. Water storage had little effect on the growth rate of the other surviving isolates. None of the isolates demonstrated morphological or biochemical changes.

Bulbil-, chlamydospore-, and conidium-forming basidiomycetes survived lyophilization and were maintained in a lyophilized state, but lyophilization killed the tropical mycelial isolates. Similar results with other hypha-forming fungi were found in previous studies (Banno et al 1979, Ellis and Roberson 1968, Haskins 1957, Haynes et al 1955, Smith and Onions 1983, 1994, Rybnikar 1985, 1995, Schipper and Bekker-Holtman 1976). Conidium-forming fungi are known to be lyophilizable (Rybnikar 1995, Smith 1984, Tan et al 1991). In the study reported here, the bulbil- and chlamydospore-forming basidiomycetes (*S. biggsiae* and *H. analogum,* respectively) also survived lyophilization. Those cultures that survived for 2 wk were able to survive for 1 yr and perhaps longer. The viability of lyophilized cultures after 1 yr of storage in the refrigerator was not substantially different from that after storage at room temperature. Although storage temperature did not apparently make a difference in our study, other research has indicated that it is preferable to store lyophilized ampoules in the refrigerator until needed (Kurtzman 1992, Malik 1992, Smith and Onions 1994).

All mycelial isolates survived cryopreservation. The currently preferred storage method is to keep microorganisms at an ultra-low temperature of -196 C in liquid nitrogen. Storage of cryovials in liquid nitrogen vapor, just above the liquid state (-150 C to - 160 C) , is an equally good method for preservation (Daily and Higgins 1973, Kurtzman 1980) and is safer when vials are rapidly thawed. Slight biochemical and metabolic activities may occur below -70 C. Recrystallization of ice may occur above - 130 C, which can easily damage fungal cells during storage (Smith and Onions 1994) because intracellular liquid water exists above - 130 C (Mazur 1984).

The results indicate that using an agar plug with stationary phase mycelial growth, a short period of cold hardening in the refrigerator, slow freezing prior to liquid nitrogen vapor storage, and rapid thawing after storage do not affect cryopreserved mycelial isolates. These isolates retained all their pre-preservation characteristics indicating that the phenotypes were preserved, which suggests that no genetic changes took place.

LITERATURE CITED

Akhtar M, Attridge MC, Myers GC, Blanchette RA. 1993. Biomechanical pulping of loblolly pine chips with selected white-rot fungi. Holzforschung 47:36–40.

Antheunisse J. 1973. Viability of lyophilized microorganisms after storage. Antonie van Leeuwenhoek 39:243–248.

Banno I, Mikata K, Sakane T. 1979. Viability of various yeasts after L-drying. IFO Res Comm 9:27–34.

Boesewinkel HJ. 1976. Storage of fungal cultures in water. Trans Br Mycol Soc 66:183–185.

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

Burdsall HH Jr, Dorworth EB. 1994. Preserving cultures of wood-decaying basidiomycotina using sterile distilled water. Mycologia 86:275–280.

Daily WA, Higgins CE. 1973. Preservation and storage of microorganisms in the gas phase of liquid nitrogen. Cryobiology 10:364–367.

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.

Ellis JJ, Roberson JA. 1968. Viability of fungus cultures preserved by lyophilization. Mycologia 60:399–405.

Garraway MO, Akhtar M, Wokoma ECW. 1989. Effect of high temperature stress on peroxidase activity and electrolyte leakage in maize in relation to sporulation of *Bipolaris maydis* race T. Phytopathology 79:800–805.

Harkin JM, Obst JR. 1973. Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. Experienta 29:281–287.

Haskins RH. 1957. Factors affecting survival of lyophilized fungal spores and cells. Can J Microbiol 3:477–485.

Haynes WC, Wickerham LJ, Hesseltine WC. 1955. Maintenance of cultures of industrially important microorganisms. Appl Microbiol 3:361–369.

Hwang S. 1960. Effects of ultra-low temperatures on the viability of selected fungus strain. Mycologia 52:527–529.

———. 1966. Long-term preservation of fungus cultures with liquid nitrogen refrigeration. Appl Microbiol 14:784–788.

———, 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.

———, Kwolek WF, Haynes WC. 1976. Investigation of ultra-low temperature for fungal cultures. III. Viability and growth rate of mycelial cultures following cryogenic storage. Mycologia 68:377–387.

Kirk TK, Burgess RR, Koning JW. 1992a. Use of fungi in pulping wood: an overview of biopulping research. In: Leatham G, ed. Frontiers in industrial mycology. New York, New York: Routledge, Chapman and Hall. p 99.

———, Lamar RT, Glaser JA. 1992b. The potential of white-rot fungi in bioremediation. In: Mongkolsuk S, ed. Biotechnology and environmental science. New York, New York: Plenum Press. p 131–138.

Kurtzman CP. 1980. Preservation of fungi by lyophilization and liquid nitrogen freezing. From the annual meeting of the Society for Industrial Microbiology, August 9–10, 1980: manual of methods. Flagstaff, Arizona: Northern Arizona University. p 4–12.

———. 1986. The ARS culture collections: present status and new directions. Enzyme Microbiol Technol 8:328–333.

———. 1992. Culture collections: methods and distribution. In: Lederberg J, ed. Encyclopedia of microbiology. Vol. 1. New York: Academic Press. p 621–625.

Lamar RT, Davis MW, Dietrich DM, Glaser JA. 1994. Treatment of a pentachlorophenol- and creosote-contaminated soil using the lignin-degrading fungus *Phanerochaete sordida*: a field demonstration. Soil Biol Biochem 26: 1603–1611.

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.

Malik KA. 1992. Freeze-drying microorganisms using a simple apparatus. J Microbiol Biotechnol 8:76–79.

Marx DH, Daniel WJ. 1976. Maintaining cultures of ectomycorrhizal and plant pathogenic fungi in sterile water cold storage. Can J Microbiol 22:338–341.

Mazur P. 1984. Freezing of living cells: mechanisms and implications. Am J Physiol 247:C125–C142.

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

Onions AHS. 1983. Preservation of fungi. In: Smith JE, Berry DR, Kristiansen B, eds. The filamentous fungi. London, England: Edward Arnold, Ltd. p 373–390.

Richter DL, Bruhn JN. 1989. Revival of saprotrophic and mycorrhizal basidiomycete cultures from cold storage in sterile water. Can J Microbiol 35:1055–1060.

Rybnikar A. 1985. Storage of lyophilized dermatophyte cultures. Acta Vet Brno 54:79–83.

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

Schipper MAA, Bekker-Holtman J. 1976. Viability of lyophilized fungal cultures. Antonie van Leeuwenhoek 42:325–328.

Smibert RM, Krieg NR. 1981. General characterization. In: Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB, eds. Manual of methods for general bacteriology. Washington, DC: American Society for Microbiology. p 411–442.

Smith D. 1984. Maintenance of fungi. In: Kirsop BE, Snell JS, eds. Maintenance of microorganisms. London, England: Academic Press. p 83–107.

———. 1993. Long-term preservation of test strains (fungus). Inter Biodeter Biodegrad 31:227–230.

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

———, ———. 1994. The preservation and maintenance of living fungi. IMI technical handbooks. 2nd ed. Wallingford, England: CAB International. 122 p.

Tan CS. 1997. Preservation of fungi. Cryptog Mycol 18:157–163.

———, Stalpers JA, van Ingen CW. 1991. Freeze-drying of fungal hyphae. Mycologia 83:654–657.

———, van Ingen CW, Talsma H, van Miltenburg LC, Steffensen CL, Vlug IJA, Stalpers JA. 1995. Freeze-drying of fungi: influence of composition and glass transition temperature of the protectant. Cryobiology 32:60–67.

———, 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.

Wickerham LJ, Flickinger MH. 1946. Viability of yeasts preserved two years: the lyophil process. Brew Dig XXI:55–60.