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Yutaka Kitamoto · Akira Suzuki · Sanae Shimada
Katsuji Yamanaka

A new method for the preservation of fungus stock cultures by deep-freezing

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Abstract Recovery of 66 fungus stock cultures including Oomycota, Zygomycota, Ascomycota, Basidiomycota, and mitosporic mycetes were examined after cryopreservation. Almost all the stock cultures remained viable when the mycelia that had grown over the sawdust medium containing 10% glycerol as the cryoprotectant (65% moisture content, W/W) were frozen rapidly at −85°C and then allow to thaw naturally at room temperature. Test stock cultures were preserved for more than 10 years by this preservation method without any programmed precooling and rapid thawing for their cryopreservation. Most of the test fungi could survive for 5 years in medium containing 10% glycerol even after alternate freezing and thawing at intervals of 6 months. When a strain of *Flammulina velutipes* was tested for mycelial growth rate and productivity of fruit-bodies after cryopreservation for 3 years, the fungus reproduced with its initial capability. These results demonstrate that the sawdust-freezing method using a cryoprotectant is expected to be a reliable and easy preservation method for fungus stock cultures.

Key words Cryopreservation · Cryoprotectant · Fungus stock preservation · Mushroom stock preservation · Sawdust-freezing method

Y. Kitamoto (✉)
Department of Biochemistry and Biotechnology, Faculty of Agriculture, Tottori University, 4-101 Minami, Koyama-cho, Tottori 680-0945, Japan
Tel. +81-857-31-5371; Fax +81-857-31-5347
e-mail: kitamoto@muses.tottori-u.ac.jp

A. Suzuki
Department of Biology, Faculty of Education, Chiba University, Chiba, Japan

S. Shimada · K. Yamanaka[1]
Mushroom Laboratory, Hokuto Corporation, Nagano, Japan

Present address:
[1]Kyoto Mycological Institute, Kyoto, Japan

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Introduction

Fungus stock cultures, especially for mushroom stock cultures, are usually kept by a subculture method, namely by serial transfer of the mycelia from stale to fresh media. The subculture method is, however, a labor-intensive procedure because it is necessary to make the transfer at least once a year. Moreover, subculturing may cause changes in certain physiological or genetic characteristics such as decrease of productivity and deterioration of quality in the fruit-bodies if applied for practical cultivation on artificial substrates (Smith 1991; Smith and Onions 1994). The preservation of fungus stock cultures has been developed to eliminate problems encountered by serial transfer of stock cultures. To decrease changes in fungus properties, fungus cultures were kept at low temperature (usually at 4°C), stocked by addition of liquid paraffin onto the culture media at 15°C, or kept under reduced oxygen tension. However, these techniques are still insufficient to resolve the problems caused during serial transfer (Hwang and San Antonio 1972; Hwang et al. 1976; Abe 1977; Iwata 1977; Yokoyama 1977; San Antonio 1978; Smith 1988, 1991; Smith and Onions 1994). On the other hand, liquid nitrogen preservation for microorganisms has proved to be one of the most reliable methods, but this method requires supplying liquid nitrogen into a vessel at a suitable interval for safekeeping (Hwang and San Antonio 1972; Hwang et al. 1976; Abe 1977; Yokoyama 1977, 1992; Smith 1988, 1991; Ohmasa et al. 1992; Smith and Onions 1994). The storage of fungus cultures below −70°C, particularly at −80°C, has been used extensively as a conventional method in Japan (Ito 1991; Ito and Nakagiri 1996) instead of storage with liquid nitrogen (Smith 1988, 1991; Smith and Onions 1994; Ohmasa et al. 1992). The preparation of fungus agar disks in a storage vessel is, however, also a delicate technique, and linear precooling and rapid thawing of fungus stocks are indispensable for reliable storage (Abe 1977; Yokoyama 1977, 1992; Smith 1988, 1991; Smith and Onions 1994).

In the present study, we conducted experiments to establish a simple and reliable freezing preservation method for

the maintenance of stocks of filamentous fungi, especially those of mushrooms.

Materials and methods

Organisms

Sixty-five fungus strains including 1 species of Oomycota, 5 species of Zygomycota, 7 species of Ascomycota, 34 species of Basidiomycota, and 9 species of mitosporic mycetes were used in the present study.

Preparation of the freeze-stock cultures

The stock cultures for deep freezing was prepared by using a basal sawdust medium. The medium contained 1.5 g beech (*Fagus crenata* Blume.) sawdust, 0.45 g rice bran, and about 3.5 ml distilled water, and the water content was adjusted to 64%. For the preparation of test media, different concentrations of cryoprotectants were added to the basal medium. The media were poured into test tubes (Pyrex), 15.5 mm in diameter and 155 mm in length, pressed to about 5 cm in height, and corked with foamed silicon rubber plugs (Shirikosen; Shinetsu Chemical, Tokyo, Japan). The cultures were autoclaved at 120°C for 10 min. To prepare the inoculum of the mycelium agar block, 16 ml agar plate medium, which consisted of 20 g glucose, hot water extract of 200 g diced potatoes, 15 g agar, and 1 l distilled water, pH 5.6, was prepared. After autoclaving at 115°C for 5 min, it was inoculated with the mycelium of the stock culture. The plate culture was incubated at 25°C in the dark for 7 days. The mycelial colony of the plate culture was cut by $\sim 3 \times 3 \times 3$ mm for the inoculum agar blocks, and an agar block was placed onto each of the test sawdust media. The cultures were incubated at 25°C to allow mycelial growth for 6 days in the dark, and they were then moved into a culture room at 25°C for 3–4 weeks until the mycelia grew over the media. The cultures were put directly in a deep-freezer set at −85°C without any program of linear precooling and stored for the designated duration. The frozen cultures were immediately thawed for 3 h at room temperature when their viabilities were determined.

Recovery test for fungus stock cultures

To test the recovery of the frozen cultures, potato dextrose agar (PDA) slant media were prepared. Eight milliliters of PDA (Difco, Detroit, USA) was poured into each test tube (Pyrex), 16.5 mm in diameter and 165 mm in length, and then autoclaved. The tubes were cooled in air to prepare slant cultures. A piece of the thawed mycelium of the test sawdust culture was inoculated onto the PDA slant and incubated at 25°C in the dark for 1 week. The thawed cultures were quickly returned to the deep-freezer to examine their viabilities after repeated alternate freezing and thawing treatments for further periods. The recovery test was conducted by using three or five frozen stock cultures of the test strain. After incubating the cultures for 7 days at 25°C, mycelial growth was observed by the naked eye. Identification of the revived cultures was done by comparing the colony morphology of the test strain with that subcultured on the PDA slant.

Productivity tests for fruit-body cultivation in two mushrooms

Flammulina velutipes (Curt.: Fr.) Singer, the commercial strain M-50 (Hokuto, Nagano, Japan), was used to test the capability of fruit-body production as a cryopreservation sample of the stock of edible mushroom strains. Five hundred grams of the substrate mixture (sawdust:rice bran = 3:1 v/v; moisture content, 65%) with or without 10% glycerol was compressed into an 850-ml polypropylene bottle, autoclaved at 120°C for 30 min, and then cooled to room temperature. For preparation of the sawdust seed spawn, about 3 g of a fragment of thawed mycelium that had been frozen at −85°C for 3 years was inoculated into the bottle and incubated at 17°C in the dark for about 4 weeks. Five grams of the seed spawn thus prepared was inoculated into the culture bottle, incubated at 17°C for 4 weeks to allow spawn running in the dark, and followed by scraping and removing the spawn having grown in the surface layer of the substrate (the "Kinkaki" treatment) to stimulate fruit-body initiation. The next cultivation process was the "Medashi" process, which was performed by incubation at 15°C at 90% relative humidity (RH) for 10 days to produce budding of fruit-bodies. This step was followed by chilling treatment (the "Yokusei" process) at 4°–6°C, 70%–80% RH, and 30–100 lx provided by daylight fluorescent lamps for 8 days. The culture was then transferred into the "Seiiku" room in which the culture was incubated at 7°–9°C and 76%–85% RH until the fruit-bodies grew to about 130 mm in height.

The culture of *Pleurotus ostreatus* (Jacq.: Fr.) Kummer subjected to cryopreservation, commercial strain Y2 (Hokuto), was also used to test changes in the productivity of mushroom stock cultures. The same sawdust medium and the same procedures for the preparation of the seed spawn as already described were applied for the cultivation test. Spawn running was carried out at 22°C and 65%–75% RH for 22–27 days. The Kinkaki treatment was done after the completion of spawn running, and the culture was transferred into the "Medashi" room where temperature and RH were adjusted to 14°C and 90%, respectively, to induce fruiting, for 12 days, followed by the Seiiku process at 15°C and 90% RH up to harvest of fruit-bodies.

The productivity of the test stock was evaluated by measuring the total weight of the fruit-bodies or by counting the number of fruit-bodies per bottle. Morphological features of fruit-bodies were observed by the methods described in The Official Report for the Characteristics of Seed Spawn of a Mushroom, "Enokitake" (1980).

Table 1. Recovery of the dikaryotic stock cultures of *Lentinula edodes* #5 grown on various media after designated times of alternate freezing and thawing

Medium	Moisture content (%)[a]	Days required for the appearance of revived mycelia after different times of alternate freezing and thawing[b]				
		1	2	3	4	5
PDA		–	–	–	–	–
Sawdust medium[c]	56	2.2 ± 0.4	7.2 ± 1.1	11.4 ± 1.1	–	–
	60	2.2 ± 0.4	2.3 ± 0.5	5.4 ± 1.1	–	–
	64	2.0 ± 0.0	4.2 ± 1.1	–	–	–
	68	2.3 ± 0.5	7.0 ± 1.2	–	–	–

–, not revived
[a] Moisture content after autoclaving
[b] Each datum is the average of five replicates with standard error
[c] The basal sawdust medium adjusted at different concentrations of moisture content

Table 2. Protective effect of different concentrations of cryoptotectants on the maintenance of dikaryotic stock cultures of *Lentinula edodes* #5 preserved by basal sawdust medium at −85°C

Cryoprotectant	Concentration (%, v/v)	Recovery of mycelia after different number of days of alternate freezing and thawing[a]				
		1	2	3	4	5
Glycerol	5	+	+	+	+	+
	10	+	+	+	+	+
	15	+	+	+	+	+
	20	+	+	+	+	+
Ethylene glycol	5	+	+	−	−	−
	10	+	+	+	+	+
Control (without protectant)		+	+	−	−	−

The stock cultures were treated with alternate freezing and thawing at 2-day intervals
+, revived; −, not revived
[a] Each datum is the result of five replicates that showed the same result

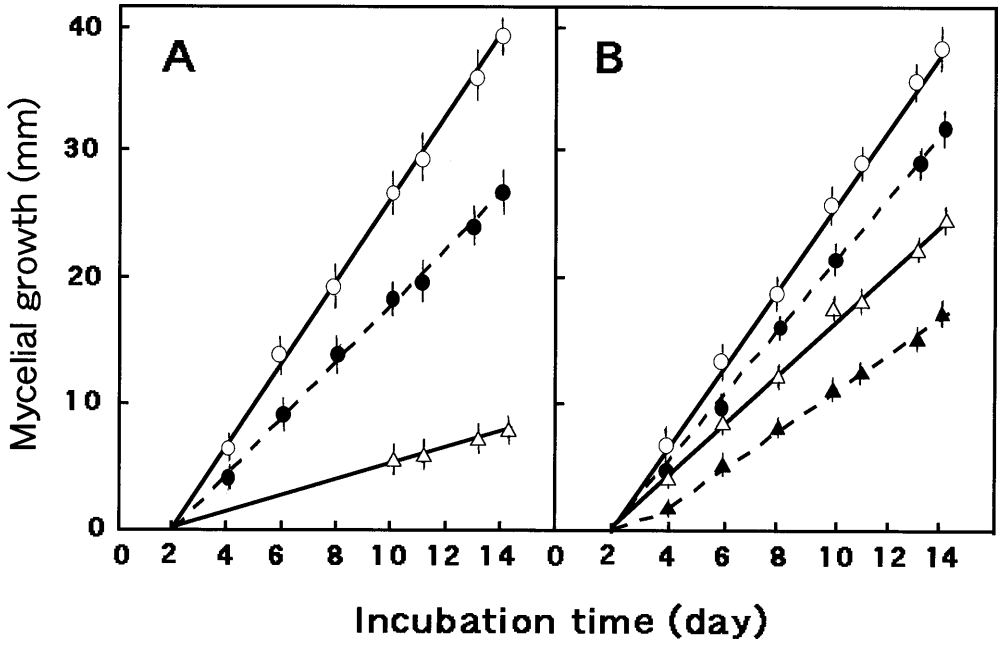

Fig. 1. Growth rates of dikaryotic mycelia of *Lentinula edodes* #5 grown on basal sawdust medium containing different concentrations of cryoprotectants. The mycelial growth of the longest and the shortest distances from the top surface of the test sawdust medium was measured with a caliper to obtain the average of the values. The mycelial growth rate of cultures in each condition was determined by averaging the values obtained by three replicates after calculating the growth rate of each culture based on this method. *Vertical line*, standard error. **A** ○, 0% ethylene glycol; ●, 5% ethylene glycol; △, 10% ethylene glycol. **B** ○, 0% glycerol; ●, 5% glycerol; △, 10% glycerol; ▲, 15% glycerol

Results and discussion

Determination of a suitable sawdust medium for deep-freezing preservation

To determine the suitable moisture content of the sawdust medium for deep-freezing preservation, the viability of stock cultures was examined after alternate freezing and thawing. As shown in Table 1, the dikaryotic stock (#5) of *Lentinula edodes* (Berk.) Pegler frozen for 7 days survived at least two repetitions of the alternate freezing and thawing treatments when the mycelia grew on sawdust media at moisture content ranging from 56% to 68% (w/w). However, the mycelium of the same fungus grown on the PDA could not survive more than one freezing and thawing treatment. When the basal sawdust media with different moisture content were adopted to cryopreservation, the time required for the first appearance of revived hyphae increased with the increment of repeated alternate freezing and thawing. Moreover, they could not survive more than three to four repeats of the preservation treatment. From these results, we estimate the optimum moisture content of the sawdust medium to be 60%–64%. Similar results were obtained for the recovery tests for *L. edodes*, #110, *F. velutipes*, M50, and *P. ostreatus*, Y2 (data not shown).

The next experiments were conducted to choose a suitable cryoprotectant to be added into the basal sawdust medium. As shown in Table 2, the dikaryotic stock of *L. edodes* survived even after five times of alternate freezing and

Table 3. Viability tests for the stock cultures of various kinds of mushrooms preserved for different periods by the sawdust-freezing method (10% glycerol)

Fungus species of Basidiomycota	Stock no.	Viability of stock cultures preserved for				
		12 months	21 months	33 months	60 months[a]	120 months[b]
Agaricus bisporus	TD-881	NE	NE	NE	+	+
Boletus pulverulentus	TD-792	NE	NE	+	NE	NE
Coprinopsis cinerea	TD-822	+	+	+	+	+
	CA-3[c]	NE	+	NE	NE	NE
Coprinopsis echinospora	581	+	+	+	NE	NE
Coprinopsis lagopides	IF030120	+	+	+	NE	NE
Coprinopsis phlyctidospora	IF030478	+	+	+	+	+
	PA-1[c]	NE	+	NE	NE	NE
Cryptoporus volvatus	TD-79	+	+	+	+	+
Entoloma clypeatum	TD-797	+	+	+	+	NE
Fistulina hepatica	F-79	+	+	+	NE	NE
Flammulina velutipes	M50	+	+	+	+	+
	J69[c]	NE	+	+	NE	NE
Ganoderma lucidum	TD-791	+	+	+	+	+
Grifola albicans	TD-793	+	+	+	+	+
Hebeloma radicosum	546	+	+	+	NE	NE
Hebeloma spoliatum	592	+	+	+	NE	NE
Hebeloma vinosophyllum	A0KI	+	+	+	NE	NE
Lentinula edodes	#5	+	+	+	+	+
Lepista nuda	NSS-61	+	+	+	−	NE
Microporus vernicipes	TD-45	+	+	+	+	+
Microporus longisporus	TD-44	+	+	+	NE	NE
Neolentinus lepideus	TD-832	+	+	NE	NE	NE
Paxillus involutus	TD-795	+	+	+	+	+
Pholiota nameko	3024	+	+	+	+	+
Pluteus leoninus	TD-20	+	+	+	+	+
Pleurocybella porrigens	HY-1	+	+	NE	NE	NE
Pleurotus ostreatus	Y2	+	+	+	+	+
Polyporus arcularius	ATCC24461	+	+	+	+	+
	69B-2[c]	NE	+	NE	NE	NE
Polyporus badius	TD-901	+	+	+	+	+
Psathyrella obtusata	TD-799	+	+	+	NE	NE
Psathyrella spadiceogrisea	TD-796	+	+	NE	NE	NE
Pycnoporus coccineus	TD-812	+	+	+	+	+
Schizophyllum commune	TD-70	+	+	+	+	+
Tephrocybe tesquorum	580	+	+	+	−	NE
Trametes versicolor	TD-17	+	+	+	+	+
Trichaptum biforme	TD911	+	+	+	NE	NE

Each datum is the result of three replicates that showed the same result
+, revived; −, not revived; NE, not examined
[a] Viability of stock cultures for 60 months by the sawdust-freezing method was determined after alternate freezing and thawing at 6-month intervals
[b] Viability of stock cultures for 120 months by the sawdust-freezing method was determined for stocks kept in a freezer without alternate freezing and thawing treatment
[c] Monokaryotic strains; all other stocks used for this experiment were dikaryotic strains

thawing on the basal sawdust medium containing 5%–20% glycerol or 10% ethylene glycol but did not survive on the medium containing 5% ethylene glycol after three times of alternate freezing and thawing. On the other hand, the basal sawdust medium (without cryoprotectant, the control) could not support the viability of the stocks for more than three repeats of alternate freezing and thawing.

The effect of addition of cryoprotectants on mycelial growth in the preservation medium is shown in Fig. 1. The growth rates of *L. edodes* decreased with the increment of concentrations of ethylene glycol and glycerol into the basal sawdust medium, but this growth-suppressing effect was weaker in the former. The fungus could not grow on the basal sawdust medium containing 15% ethylene glycol. The density of the revived mycelium after cryopreservation might not be affected by the addition of 5%–10% glycerol but became sparser after the addition of 15% glycerol and 5%–10% ethylene glycol.

It is indicated that glycerol is a more effective cryoprotectant than ethylene glycol. The superiority of glycerol as the cryoprotectant has been reported in many liquids and agar media used for storage by freezing (Yokoyama 1977; Smith 1988, 1991; Ohmasa et al. 1992; Smith and Onions 1994). We concluded that the sawdust medium with 10% glycerol might produce the best result in our cryopreservation method.

Recovery of fungus stock cultures preserved by the sawdust-freezing method

Table 3 shows the results of sawdust cryopreservation test for the dikaryotic mycelial stocks of 34 mushrooms in Basidiomycota. Test stock cultures were preserved for more than 10 years by this preservation method without any programmed precooling and rapid thawing for their cryopreservation. Most of the test fungi could survive for 5 years in the medium containing 5%–10% glycerol even after alternate freezing and thawing at intervals of 6 months. Ectomycorhizal fungi such as *Boletus pulverulentus* Opat., *Entoloma* sp. (Agerer and Waller 1993), and *Hebeloma* spp. (Sagara 1995) could be preserved reliably, as well as wood-rotting fungi and saphrobic fungi by the sawdust-freezing method. The monokaryotic mycelium strains of

Table 4. Viability tests for stock cultures of various kinds of molds preserved for different periods by the sawdust-freezing method (10% glycerol)

Fungus species	Stock no.	Viability of stock cultures preserved for					
		3 days	7 months	20 months	36 months	60 months[a]	120 months[b]
Oomycota							
Phytophthora infestans	AKU 3875	+	+	NE	NE	NE	NE
Zygomycota							
Helicostylum nigricans	IF0 8091	+	+	+	NE	NE	NE
Mortierella humicola	IF0 8289	+	+	+	NE	NE	NE
Mucor racemosus	IF0 4581	+	+	+	NE	NE	NE
Phycomyces nitens	IF0 9422	+	+	+	NE	NE	+
Syncephalis sphaerica	ATCC 36849	+	+	+	NE	NE	NE
Ascomyccota							
Arthroderma uncinatum	AKU 3830	+	+	+	NE	NE	NE
Bipolaris oryzae	TF-883	+	+	+	NE	NE	+
Emericella nivea	TF-884	+	+	NE	NE	NE	NE
Eurotium chevalieri	TF-856	+	+	+	NE	NE	NE
Gibberella fujikuroi	FG-1	+	+	+	NE	NE	NE
Monascus anka var. *rubellus*	AKU 3503	+	+	+	NE	NE	NE
Neurospora crassa	IF0 6068	+	+	+	+	+	+
Mitosporic mycetes							
Aspergillus niger	TF-798	+	+	+	+	+	+
Aspergillus oryzae	TF-791	+	+	+	NE	+	+
	IAM 2649	+	+	+	NE	+	+
	IAM 2704	+	+	+	NE	+	+
	IAM 2736	+	+	+	NE	+	+
	IF0 4348	+	+	+	NE	+	+
	NK	+	+	+	NE	+	+
Aspergillus sojae	FA-29	+	+	+	NE	+	+
Beauveria bassiana	AKU-3876	+	+	+	NE	NE	NE
Botrytis cinerea	TF-851	+	+	+	NE	NE	NE
Cladosporium herbarum	IAM 5059	+	+	+	NE	NE	NE
Fusarium culmorum	AKU 3702	+	+	+	NE	+	+
Penicillium chrysogenum	IF0 4897	+	+	+	NE	+	+
Trichoderma harzianum	TMI 60622	+	+	+	NE	+	+

Each datum is the result of three replicates that showed the same result
+, revived; −, not revived; NE, not examined
[a] Viability of stock cultures preserved for 60 months by the sawdust-freezing method was determined after alternate freezing and thawing at 6-month intervals
[b] Viability of stock cultures for 120 months by the sawdust-freezing method was determined for stocks kept in a freezer without alternate freezing and thawing treatment

Table 5. Yields and shape of fruit-bodies of two mushrooms by the sawdust-freezing method and subculture method

Fungus species	Stock no.	Preservation method[a]	Yield (g/bottle)[b]	Number of fruit-bodies[c]	Pileus diameter (mm)	Stipe length (mm)	Stipe diameter (mm)
Flammulina velutipes	M50	Freezing	127 ± 15	512 ± 31	9.8 ± 1.1	135 ± 11	2.6 ± 0.6
		Subculture	137 ± 13	511 ± 36	8.8 ± 1.4	126 ± 9	3.0 ± 0.7
Pleurotus ostreatus	Y2	Freezing	110 ± 9	ND	ND	ND	ND
		Subculture	109 ± 7	ND	ND	ND	ND

Each datum is the average of 10 culture bottles with standard error
ND, not determined
[a] Freezing, frozen at −85°C for 3 years; subculture, stored at 2–3°C and subcultured three times during 3 years of preservation
[b] Total fresh weight of fruit-bodies per culture bottle
[c] Number of fruit-bodies per culture bottle

four test mushrooms in Basidiomycota could be preserved by adopting of this preservation method, at least for more than 21 months (Table 3). Moreover, it showed that various species of molds in Oomycota, Zygomycota, Ascomycota, and mitosporic mycetes could be also reliably preserved by the sawdust-freezing method (Table 4). It has been reported that the viabilities of the stock cultures differed from one another even when they were derived from the same species (Yokoyama 1977; Smith 1991; Smith and Onions 1994). These results suggest that the sawdust-freezing method is expected to be a suitable way to achieve reliable preservation of various kinds of fungus stock cultures.

Evaluation of yield and quality of fruit-bodies of mushroom stock cultures by the sawdust-freezing method

Hwang and San Antonio (1972) reported that the stock cultures of *Agaricus bisporus* (J. Lange) Imbach could be preserved by freezing at −196°C in 10% glycerol without any significant changes in the productivity of fruit-bodies for at least 2 years and as safely as by subculture at 5°C. Ohmasa et al. (1992) reported in a similar study that the stock culture of *P. ostreatus* could be preserved without any significant changes in the productivity of fruit-bodies for at least 4 years by freezing at −85° in either one of two cryoprotectants, 10% glycerol or 5% dimethyl sulfoxide (DMSO), and as safely as by subculture at 10°C. However, the yield of fruit-bodies of a stock culture in *F. velutipes* varied among the cultures derived from the same mycelial agar disks of the same strain preserved in 10% glycerol for 7 years at −85°C (Ohmasa et al. 1996).

We also examined the effect of our cryopreservation treatment on the productivity of fruit-bodies, and their quality was examined by sawdust cultivation tests for two edible mushrooms, *F. velutipes* and *P. ostreatus*. As shown in Table 5, the yield of fruit-bodies of both mushrooms using spawns after sawdust cryopreservation for 3 years did not show any obvious difference from corresponding results using spawns that were kept for 3 years by three subculture cycles. In the former species, it was also confirmed that the cultures did not show any change in the shape of fruit-bodies when their inoculum stock cultures were frozen at −85°C with natural freezing treatment. The mycelia grown on the sawdust cultures are easy to handle by the small changes in their texture even after repeated freezing and thawing. Further, it does not require labor-intensive exercises, such as the linear precooling and rapid thawing (Abe 1977; Yokoyama 1977, 1992; Smith 1988, 1991; Ito 1991; Smith and Onions 1994; Ito and Nakagiri 1996; Ohmasa et al. 1992, 1996) that is indispensable for the usual storage by deep-freezing for reliable preservation. These results suggest that the sawdust-freezing method with usage of a cryoprotectant is one of the most suitable methods compared to other freezing methods using agar disks.

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References

Abe S (1977) Preservation methods in filamentous fungi (I) – *Asperugillus*, *Penicillium* and Ascomycetes, Phycomycetes In: Nei T (ed) Methods in preservation of microorganisms (in Japanese). University of Tokyo Press, Tokyo, pp 291–315

Agerer R, Waller K (1993) Mycorrhizae of *Entoloma saepium*: parasitism or symbiosis? Mycorrhiza 3:145–154

Hwang SW, San Antonio JP (1972) Stability of spawn stocks of the cultivated mushroom after 26 months liquid nitrogen refrigeration (−160°C to −196°C). Mushroom Sci 8:35–42

Hwang SW, 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

Ito T (1991) Frozen storage of fungal cultures deposited in the IFO culture collections. IFO Res Commun 15:119–128

Ito T, Nakagiri A (1996) Viability of frozen cultures of basidiomycetes after fifteen-year storage. Microbiol Culture Coll 12:67–78

Iwata K (1977) Preservation methods in pathogenic fungi. In: Nei T (ed) Methods in preservation of microorganisms (in Japanese). University of Tokyo Press, Tokyo, pp 265–274

Ohmasa M, Abe Y, Babasaki K, Hiraide M, Okabe K (1992) Preservation of cultures of mushrooms by freezing. Trans Mycol Soc Jpn 33:467–479

Ohmasa M, Tsunoda M, Babasaki K, Hiraide M, Harigae H (1996) Fruit-body production of test cultures of *Flammulina velutipes* preserved for seven years by freezing at three different temperatures. Mycoscience 37:449–454

Sagara N (1995) Association of ectomycorrhizal fungi with decomposed animal wastes in forest habitats: a cleaning symbiosis? Can J Bot 73(suppl 1):S1423–S1433

San Antonio JP (1978) Stability of spawn stocks of the cultivated mushroom stored for nine years in liquid nitrogen (−160°C to −196°C). Mushroom Sci 10(part I):103–113

Smith D (1988) Culture and preservation. In: Hawksworth DL, Kirsop BE (eds) Living resources for biotechnology: filamentous fungi. Cambridge University Press, Cambridge, pp 75–79

Smith D (1991) Filamentous fungi. In: Kirsop BE, Doyle A (eds) Maintenance of microorganisms and cultured cells: a manual of laboratory methods, 2nd edn. Academic Press, New York, pp 133–159

Smith D, Onions AHS (1994) The preservation and maintenance of living fungi, 2nd edn. CAB International, Wallingford

The official report for the characteristics of seed spawn of a mushroom, "Enokitake" (1980) (in Japanese). The Mushroom Seed Spawn Association of Japan, Tokyo, pp 3–12

Yokoyama T (1977) Preservation methods in filamentous fungi (II). Basidiomycetes and Deuteromycetes. In: Nei T (ed) Methods in preservation of microorganisms (in Japanese). University of Tokyo Press, Tokyo, pp 317–353

Yokoyama T (1992) Preservation of mycelia. In: Furukawa H (ed) Mushroom science (in Japanese). Kyoritsushuppan, Tokyo, pp 230–237