Storage and Preservation of Temperate Mushroom Cultures, *Agaricus Bisporus* and *Pleurotus Florida*

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Abstract Two temperate mushroom cultures namely *Agaricus bisporus* (U-3) and *Pleurotus florida* (PAU-5) were evaluated for their physiological (linear growth and biomass production), biochemical (β -1,4 endoglucanase production) and fruiting behaviour after preservation in 10% (v/v) glycerol and storage at room temperature (25–35°C), –20°C and –196°C for 6 months with the objective of establishing the recovery/changes in these fungi after storage. Studies indicated that the viability and recovery of *A. bisporus* and *P. florida* is affected by the storage conditions. Both the fungi could be best stored in liquid nitrogen for longer durations but for regular use, conventional sub-culturing was appropriate.

Keywords Mushroom cultures · Glycerol · Growth · Liquid nitrogen · Preservation

Fungi are one of the longest preserved organisms by sub-culturing methods and have been preserved since 1895 [1]. Cultures of known fungi have been stored either as spores or as vegetative mycelia. A wide variety of methods like frequent sub-culturing, storage in mineral oil or paraffin wax, storage in sterile distilled water, lyophilization, deep freezing etc. are available for conservation of fungal cultures [2]. Cryopreservation is a process where cells or whole tissues are preserved by cooling to low sub-zero temperatures by adding cryoprotectants which act as anti-freeze by lowering the freezing temperature and increasing

the viscosity. Cryopreservation of cultures is considered as the most acceptable method of preservation [3].

Mushroom production is a profitable industry and a wide range of edible fungi have been successfully cultivated. Pure culture maintenance is important for producing fruiting bodies with excellent flavour, texture and colour along with their resistance to pests and diseases. Stock cultures are routinely maintained on a suitable solid substrate using short term periodic transfer. However, long-term storage involves retardation of cellular metabolism.

The present study was undertaken to study the effect of preservation on the physiological, biochemical and morphological characteristics of *Agaricus bisporus* and *Pleurotus florida* mushroom mycelia in relation to its purity and the fruiting efficiency by storage at different temperatures.

A. bisporus (U-3) and *P. florida* (PAU-5) were procured from germplasm collection bank of Department of Microbiology, Punjab Agricultural University, Ludhiana and were grown on PDA at $25 \pm 2^\circ\text{C}$ till the mycelia had covered the surface of PDA plates. Mycelial disks (5 mm) were punched out using a sterile cork borer under aseptic conditions and 10–12 disks of each strain were transferred into vials containing 10% (v/v) glycerol. The vials were then stored for 6 months at room temperature (25–32°C), –20°C and in liquid nitrogen (–196°C). The cultures were initially stored at 4°C for 2 h, followed by 2 h at –20°C and at –80°C and then plunged into liquid nitrogen for long term storage. The cultures were recovered at monthly intervals and inoculated on 90-mm agar plates. Cultures stored at –20°C were thawed to room temperature for 30 min before inoculation on the media. The cultures stored at –196°C were first kept for 2 h each at –80 and –20°C and then allowed to thaw at room temperature and subsequently used for growth studies and enzyme estimation.

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For growth studies, the mycelial bits of culture from different temperatures were placed aseptically in the center of PDA plates, incubated at $25 \pm 2^\circ\text{C}$ and growth measured as colony diameter (two orthogonal diameters per colony) was recorded after an interval of 4 days for *A. bisporus* and 3 days for *P. florida*, respectively. Average of three agar plates was recorded as growth in mm.

For biomass studies, culture bit was inoculated in 100 ml of complete yeast extract medium (CYM) broth [4]. After 5 days (U-3) and 3 days interval (PAU-5), mycelial biomass of three replicate flasks of each treatment was filtered on a pre-weighed Whatman filter paper no. 1. The filter paper along with mycelial biomass was dried at 60°C for 8 h and weighed.

For qualitative assay of β -1,4 endoglucanase, modified Mushroom Minimal Medium (glucose replaced by 0.5% Carboxymethyl Cellulose, CMC) was inoculated with a 5 mm bit of culture. After 10 days (U-3) and 7 days (PAU-5) of incubation at $25 \pm 2^\circ\text{C}$, three replicate Petri plates for each treatment were stained with Congo red dye (1 mg/ml) for 15 min and then destained with 1 M NaCl solution for 15 min to observe a halo zone. The colony diameter (C) and the zone diameter (Z) were recorded from two perpendicular planes to determine mean Z/C ratio. Carboxymethyl Cellulose degrading activity (β -1,4-endoglucanase) was measured for three replicates of each treatment by estimation of the reducing sugars released during incubation of the mycelium with Mushroom Minimal Medium [5]. The reducing sugars were estimated using DNS reagent [6]. The total protein content was also estimated [7] and enzyme specific activity was calculated as mg of reducing sugar produced/min/ml of enzyme extract/mg crude protein.

A. bisporus and *P. florida* spawn was prepared from cultures preserved at different temperatures on wheat grains inoculated with mycelial agar bit and incubated at $25 \pm 2^\circ\text{C}$ till the mycelia completely covered the grains. Cultivation of *A. bisporus* was carried out on wheat straw based compost [8] filled in bags used @ 1.5 kg per bag and spawned @ 0.6% by weight of wet compost. The bags were kept in growing room for mycelium proliferation and casing was done after 15 days of spawning. After 10–12 days of casing, pinheads began to appear which developed into mature fruit bodies within 5–6 days. Ten replicate bags were maintained for each treatment. For cultivation of *P. florida*, wheat straw was wetted to 70% moisture, filled in bags @ one kg wet straw per bag and spawned @ 3.5% (w/w). The bags were incubated in growing house and opened on completion of spawn run. Bags were watered daily to obtain fruit bodies. Ten replicate bags were maintained for each treatment.

The results of the study showed that the growth of mycelium on preservation of *A. bisporus* at -196°C , was found optimal up to 5 months of storage. Similar results

have also been found in which liquid nitrogen freezing of *A. bisporus* strains followed by subsequent thawing did not impair the growth capacity [9]. The cultures stored at room temperature showed a significant decline in linear growth after 12 days of incubation when storage was prolonged for 5 months but growth rate was static after 16 days of incubation. In general, the growth was rapid after the culture overcame its lag period. Fortnightly subculturing of *A. bisporus* (U-3) showed a rapid and persistent growth @ 3.9 mm/day when grown as control for 16 days.

When *P. florida* (PAU-5) was preserved in glycerol and stored in deep freezer at -20°C or in liquid nitrogen at -196°C , statistically no difference in colony diameter was seen on PDA after 12 days. An increase in colony diameter @ 5.8 mm/day with increase in incubation period was observed when *P. florida* was preserved in glycerol at room temperature for 5 months. Similar results on mycelial growth of *Flammulina velutipes* cultures stored in liquid nitrogen for 7 years have been recorded [10].

It was found that in glycerol at -196°C , there was a delay in growth of mycelium of *A. bisporus* till 5 days of incubation. Preservation of *A. bisporus* in glycerol at -20°C showed a decline in biomass up to 18.5% in the lot studied after 5 months of preservation. However, there was a decrease of up to 40% in biomass with an increase in preservation period of *A. bisporus* stored at room temperature. In comparison to the growth of *A. bisporus* on agar medium, which showed 100% viability, the percent viability in glycerol at -20 and -196°C was found to decrease with increase in storage period (Table 1). Similar studies showed that the recovery of frozen mycelium of various mushroom varieties studied by them was delayed compared to the control group, which was not frozen [11]. *P. florida* (PAU-5) stored in glycerol at -196°C showed a marginal decrease in per cent viability (from 100 to 87.5%) after 5 months of preservation. Preservation of *P. florida* at -20°C showed a decrease in viability (up to 75%) after 5 months. However, as compared to control, preservation at room temperature showed only 50% viability (Table 1).

β -1,4 Endoglucanase estimation for *A. bisporus* preserved in glycerol at -196°C showed higher values of Z/C ratio (1.35, 1.35 and 1.32) for up to 6 months of storage. Cultures preserved in glycerol at -20°C showed lower Z/C ratio (1.32, 1.26 and 1.27). The cultures preserved at room temperature showed an increase in Z/C ratio @0.06/day after 20 days with increase in storage period. A Z/C ratio of 0.11/day was observed when cultures of *P. florida* stored at -196°C and as control were subjected to qualitative enzyme estimation. Least Z/C ratio of 1.20 was observed in case of *P. florida* cultures preserved in glycerol at room temperature after 14 days (Table 2). β -1,4 Endoglucanase activity was measured along with total protein content to obtain enzyme specific activity. *A. bisporus* preserved in

Table 1 Effect of preservation at various temperatures after periodic intervals on biomass production of *Agaricus bisporus* (U-3) and *Pleurotus florida* (PAU-5)

Incubation period (days)	Biomass in mg/100 ml (% increase/decrease w.r.t. 1st month)												CD (5%)
	Room temperature			−20°C			−196°C			Control			
	I ^a	II ^b	III ^c	I	II	III	I	II	III	I	II	III	
<i>Agaricus bisporus</i>													
5	ND	ND	ND	60	55 (−8.3)	25 (−58.3)	ND	ND	ND	45	35 (−22.2)	30 (−33.3)	A: NS B: 13.8 AB: NS
10	45	32	ND	115	100 (−13.0)	95 (−17.4)	55	55 (0.0)	65 (+18.2)	105	95 (−9.5)	85 (−19.0)	A: NS B: 32.3 AB: NS
15	85	76 (−10.6)	55 (−35.3)	130	120 (−7.7)	105 (−19.2)	205	160 (−21.9)	145 (−29.2)	230	210 (−8.7)	210 (−8.7)	A: NS B: 66.9 AB: NS
20	175	160 (−8.6)	105 (−40.0)	270	235 (−12.9)	220 (−18.5)	275	235 (−14.5)	235 (−14.5)	305	275 (−9.8)	300 (−1.6)	A: NS B: 45.4 AB: NS
% Viability	66.7	25	25	75	66.7	66.7	75	66.7	66.7	100	100	100	
<i>Pleurotus florida</i>													
4	ND	ND	ND	25	ND	ND	40	30 (−25.0)	30 (−25.0)	40	30 (−25.0)	30 (−25.0)	A: NS B: 12.3 AB: NS
7	60	45 (−25.0)	25 (−58.3)	90	55 (−38.9)	35 (−61.1)	55	55 (0.00)	50 (−9.09)	60	60 (0.0)	75 (+25.0)	A: NS B: 21.0 AB: NS
10	105	105 (00.0)	95 (−9.52)	175	155 (−11.4)	155 (−11.4)	190	90 (−52.6)	115 (−39.5)	165	185 (+12.1)	195 (+18.9)	A: NS B: 35.9 AB: 62.3
13	200	195 (−2.5)	230 (+15.0)	205	235 (+14.6)	200 (−2.4)	260	205 (−21.1)	190 (−26.9)	235	210 (−10.6)	255 (+8.5)	A: NS B: NS AB: NS
% Viability	50	50	50	100	75	75	100	100	87.5	100	100	100	

Medium used: complete yeast extract medium (CYM)

Control: Slant cultures stored at 25°C

A storage period, B temperature treatments, AB storage period × temperature treatments, ND not determined

^a 1-Month storage^b 3-Month storage^c 5-Month storage

Table 2 Qualitative estimation for endoglucanase activity of *Agaricus bisporus* (U-3) and *Pleurotus florida* (PAU-5) at periodic intervals after preservation (in glycerol) and storage at different temperatures

Incubation period (days)	Zone/Colony (Z/C) Ratio											
	Room temperature			−20°C			−196°C			Control		
	I ^a	II ^b	III ^c	I	II	III	I	II	III	I	II	III
<i>Agaricus bisporus</i>												
10	1.12	1.17	1.00	1.22	1.21	1.17	1.35	1.39	1.35	1.44	1.33	1.30
20	1.19	1.17	1.15	1.32	1.26	1.27	1.35	1.35	1.32	1.49	1.41	1.39
<i>Pleurotus florida</i>												
7	1.29	1.21	1.20	1.42	1.36	1.39	1.48	1.45	1.43	1.54	1.45	1.48
14	1.35	1.27	1.20	1.47	1.46	1.31	1.60	1.55	1.51	1.59	1.50	1.52

Medium used: mushroom minimal medium (MMM)

Control: slant cultures stored at 25°C

^a 2-Month storage^b 4-Month storage^c 6-Month storage

glycerol in liquid nitrogen (−196°C) as well as the control showed better growth in terms of enzyme production irrespective of the storage period for up to 6 months. However, when the cultures were preserved in glycerol either in liquid nitrogen at −196°C, in deep freezer at −20°C or at room temperature; a decline in enzyme specific activity @ 13.0, 7.6 and 80.2% respectively, was observed after 20 days of incubation. When the cultures were preserved in glycerol at room temperature, there was a decline in specific activity values from 1.08 to 0.38 after storage of 6 months. Significant variation in specific activity was seen after 7 and 14 days of incubation (Table 3).

A. bisporus preserved in glycerol at three different temperatures along with the control was used to prepare spawn on wheat grain based substrate. The fruiting test was conducted in growth chamber at Mushroom Research Complex, PAU, Ludhiana. The fruiting was observed in all the treatments except in the one stored in glycerol at room temperature. However, it was seen that *A. bisporus* (U-3) preserved in glycerol and stored at −196°C took nearly same time for fruiting as taken by control (27 and 25 days respectively) while cultures preserved at −20°C took maximum time (30 days) for pinhead formation. These observations showed that the *A. bisporus* (U-3) culture

Table 3 Variation in endoglucanase enzyme specific activity of *Agaricus bisporus* (U-3) and *Pleurotus florida* (PAU-5) at periodic intervals after preservation (in glycerol) and storage at different temperatures

Incubation period (days)	Specific activity (IU ^a /mg crude protein)											
	Room temperature			−20°C			−196°C			Control		
	I ^b	II ^c	III ^d	I	II	III	I	II	III	I	II	III
<i>Agaricus bisporus</i>												
10	0.31	0.15	0.09	0.27	0.24	ND	0.33	0.33	0.27	0.37	0.32	0.32
20	1.06	0.77	0.21	1.05	0.96	0.97	1.23	1.21	1.07	1.30	1.19	0.91
<i>Pleurotus florida</i>												
7	0.82	0.79	0.67	1.25	0.91	0.75	1.29	1.22	1.22	1.49	1.40	1.38
14	1.08	0.73	0.38	1.64	1.25	1.19	2.25	2.25	2.17	2.29	2.15	2.10

Medium used: mushroom minimal medium (MMM)

Control: slant cultures stored at 25°C

ND not determined

^a mg of reducing sugar produced/min/ml of enzyme extract^b 2-Month storage^c 4-Month storage^d 6-Month storage

Table 4 Fruiting/viability test of *Agaricus bisporus* (U-3) and *Pleurotus florida* (PAU-5) stored under different sets of conditions

Temperature treatments	<i>Agaricus bisporus</i>		<i>Pleurotus florida</i>	
	Days for pinning ^a	Fruiting potential	Days for pinning ^a	Fruiting potential
Room Temperature	—	—	64	+
−20°C	30	+	64	+
−196°C	27	+	60	+
Control (25°C)	25	+	57	+

Agaricus bisporus: date of sowing 2 Aug 2007, date of casing 17 Aug 2007, spawning @ 0.6%, 1.5 kg compost/bag

Pleurotus florida: date of sowing 12 June 2007, spawning @ 10%, 1 kg wet wheat straw/bag

^a Average of five replicates

stored in glycerol at −196°C gave results at par with control.

The cultures of *P. florida* obtained from all the treatments showed fruit body formation. However, it was seen that cultures preserved in glycerol at room temperature and −20°C took longer time on an average for both spawn run (48 days) and pinhead formation (64 days) as compared to time taken by control (45 and 57 days respectively) and cultures stored at −196°C (47 and 60 days respectively).

There was no difference in shape, color or other morphological characteristics of fruit bodies formed by cultures stored at various temperatures indicating that preservation of mushroom cultures in cryoprotective agents at room temperature, in deep freezer at −20°C or in liquid nitrogen at −196°C did not alter the morphology of cultures preserved (Table 4). Similar results in which no apparent change in physiological or morphological characteristics of other mushrooms stored in liquid nitrogen for 10 years have been obtained [12, 13]. The studies showed that the mycelia stored at room temperature or lower temperature are inactivated on prolonged storage to a greater extent than those stored at −20°C or at −196°C. Therefore, temperate mushroom cultures like *A. bisporus* and *P. florida* can conveniently be stored in glycerol and preserved in liquid nitrogen for longer periods. Routine sub-culturing of cultures is acceptable for short duration storage at their optimum temperatures.

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