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Effect of trehalose on the spawn storage in some edible mushroom fungi (2): Effect on preservation in the freezer

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Abstract We describe the effects of trehalose on spawn storage in a home freezer (average temperature, −16°C) where edible fungi usually do not survive. When the mycelia of *Lentinula edodes* were stored in a freezer for 3 days, the survival rate of mycelia cultivated on 2% glucose medium was 30%, whereas those on media containing 2% and 5% trehalose were 50% and 60%, respectively. Addition of trehalose to the culture was more effective in *Pleurotus ostreatus*. These results suggest that trehalose played the role of a stress protectant against freezing, because the mycelia cultured on a trehalose medium grew more rapidly and produced more fruiting bodies compared to those cultured on glucose.

Key words Basidiomycetes · Edible mushroom fungi · Fruiting body growth · Spawn storage · Trehalose

The preservation of mushroom cultures, that is, avoiding changing their cultivation characteristics, is very important for mushroom breeding and cultivation. The preservation of culture stocks by freezing with liquid nitrogen (LN method) is known to be a useful method for many kinds of mushrooms (Jodon et al. 1982; Ohmasa et al. 1996). However, cultivators generally store the spawn in refrigerators, as described previously (Terashita et al. 2002).

We have previously reported the effects of trehalose on growth of three kinds of basidiomycetes [*Lentinula edodes* (Barkeley) Pegler, *Pleurotus ostreatus* (Jacq.: Fr.) Kummer, and *Flammulina velutipes* (Curt. ex Fr.) Sing.] preserved in a refrigerator (Terashita et al. 2002). The survival rate of *L. edodes* mycelia cultured on trehalose media increased considerably compared with that on a glucose medium when the mycelia were stored at 4°C with less than 20% relative humidity. Trehalose was the most effective on mycelial growth of *P. ostreatus* of the three strains tested.

The roles of trehalose as a stress protectant have been studied for *Coprinus psychromorbidus* Redhead & Traquair and *Schizophyllum commune* Fr.: Fr. by Tan and Stalpers (1991) and for bakers' yeast by Hirasawa et al. (2001). Roser (1991) described that trehalose stabilized the cytoplasmic membrane and liposome. Wiemken (1990) and Rudolph and Crowe (1985) have referred to the beneficial effect of trehalose for stress loads.

In this article, we describe the effect of trehalose on spawn storage in a home freezer (average temperature, −16°C) where edible fungi usually do not survive.

L. edodes Mori no. 465 (commercial strain), *F. velutipes* IFO 7777, and *P. ostreatus* Kitamura (commercial strain) were used in this study as previously described (Terashita et al. 2002). These fungal strains were transferred annually on medium including potato extract and 2% dextrose agar (PDA).

The mycelial disk of each strain was inoculated on the center of PDA in a 9-cm-diameter Petri dish and incubated at 24°C for 10 days; mycelial disks were then punched out with a cork borer (10 mm in diameter). The mycelial disks of strains were each put on the center of a 9-cm-diameter Petri dish containing one of the following agar media – PDA (containing 2% glucose as growth substrate, control), PDTA (containing 1% glucose and 1% trehalose), PTA-2 (containing 2% trehalose), and PTA-5 (containing 5% trehalose) – and incubated at 24°C for 10 days. The media each consisted of a carbohydrate, hot water extract of 200 g diced potatoes, 15 g agar, and 1 l distilled water; pH was 5.6.

The mycelia grown on each medium containing trehalose were punched out again with a 10-mm cork borer, and the mycelial disks were put directly in the freezer at −10° to −19°C (average temperature, −16°C) for the designated duration (1–10 days) without any program of linear precooling. To test the recovery of the frozen cultures, PDA slant

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media were prepared. After a piece of the thawed mycelium (room temperature, 20°–25°C) was inoculated onto PDA slant medium and incubated at 24°C for 10 days, mycelial growth was observed by the naked eye. The recovery test was conducted by using ten frozen stock cultures of the test strain.

For experiments on mycelial growth and fruiting body formation, the mycelial disks of *L. edodes* stored in the freezer were collected at similar intervals as above. The disks were thawed at room temperature (20°–25°C), and inoculated in a glucose peptone yeast exract (GPY) liquid medium (50 ml/vessel) (Terashita et al. 2002) in 250-ml polycarbonate vessels (110 mm in diameter, 70 mm in height), then incubated at 24°C for 30 days. Fruiting body formation was induced by adjusting the temperature to 15°C under about 100–200 lx light. When the fruiting bodies were matured, they were harvested.

The dry weight of vegetative mycelia was measured 30 days after inoculation. Mycelial mats were separated from the medium by filtration and washed three to five times with distilled water. The washed mycelia were dried at 80°C for 24 h and weighed. The fruiting bodies were harvested after their maturation, and dry weights were measured after treatment at 80°C for 24 h. Data represented the average of triplicate experiments with five polycarbonate vessels per experiment.

Table 1 shows the results of the mycelial growth test of *L. edodes*, *F. velutipes*, and *P. ostreatus*. For *L. edodes*, the number of revived mycelia after storing for 3 days in the freezer was 3 of 10 (PDA and PDTA medium), 5 of 10 (PTA-2 medium), and 6 of 10 (PTA-5 medium), respectively. The mycelia that were grown on the glucose medium, and then stored for 10 days in the freezer, did not revive. On the other hand, when the mycelia were grown on trehalose media, 1 or 2 of 10 slants revived.

Survival rates of the mycelia of *F. velutipes* (Table 1) were increased by adding trehalose to the medium. When the mycelia grown on PTA-2 or PTA-5 were stored for 10 days in the freezer, 1 of 10 slants revived. However, except for 10 days freezing, the survival rate of this fungus was higher than those of *L. edodes*.

P. ostreatus was the most freeze-tolerant organism in this study (see Table 1). The survival rates of the mycelia cultured on PDA (control) were found to be lower than those cultured in a trehalose medium such as PDTA, PTA-2, and PTA-5, and the mycelia from those three media grew faster than that from PDA. Eight or 9 of 10 slants inoculated with mycelia from the trehalose medium survived after the vegetative mycelia were stored in the freezer, even for 10 days. The effect of trehalose on freeze stress was even more marked in *P. ostreatus* than when kept in a refrigerator (4°C) (Terashita et al. 2002). *P. ostreatus* stored in a refrigerator grew less than that stored in a freezer. This difference between refrigerator and freezer treatment affecting microorganisms was expected.

Table 2 shows the yields of *L. edodes* mycelia cultured in trehalose media after storing the spawns. Mycelia grown on PDA showed about 10% growth after 3 days of freezing. Additionally, the mycelia did not grow after freeze treatment for 10 days. However, the growth rate of mycelia grown on PTA-2 showed about 49% growth for 1 day of storage in the freezer, about 29% for 3 days, and about 8% for 10 days.

Table 3 shows the effect of spawn storage in the freezer on the fruiting body formation of *L. edodes*. The fruiting body yield of freeze-stored spawns was increased by the

Table 1. Effect of trehalose on the preservation of spawn of *Lentinula edodes*, *Flammulina velutipes*, and *Pleurotus ostreatus*

Number of survival test tubes for mycelial growth/10 tubes tested

Days for freezing of spawn	1				3				5				10			
Incubation days after freezing	1	3	5	10	1	3	5	10	1	3	5	10	1	3	5	10
L. edodes																
PDA (control)	0	2	3	8	0	0	2	3	0	0	0	1	0	0	0	0
PDTA	0	2	4	9	0	0	3	3	0	0	2	2	0	0	0	1
PTA-2	0	5	9	10	0	1	3	5	0	0	2	2	0	0	1	2
PTA-5	0	4	6	10	0	1	5	6	0	1	1	2	0	0	1	2
F. velutipes																
PDA (control)	0	5	6	8	0	1	6	6	0	1	4	6	0	0	0	0
PDTA	0	7	9	9	0	1	6	6	0	3	5	6	0	0	0	0
PTA-2	0	5	9	10	0	1	4	5	0	3	5	8	0	0	0	1
PTA-5	0	3	7	8	0	3	5	6	0	3	5	7	0	0	0	1
P. ostreatus																
PDA (control)	9	10	10	10	5	9	10	10	1	2	5	9	0	2	2	2
PDTA	8	10	10	10	7	9	10	10	2	6	8	9	0	3	8	8
PTA-2	10	10	10	10	9	10	10	10	8	10	10	10	0	4	8	9
PTA-5	9	10	10	10	9	10	10	10	9	10	10	10	1	6	8	8

Survival ratio of mycelial growth is given by number of test tubes with mycelial growth/10 tubes tested. The vegetative mycelia of *L. edodes*, *F. velutipes*, and *P. ostreatus* for survival tests were grown on potato trehalose agar media (0%–5%). Then, the mycelial disks were prepared by using a cork borer. The mycelial disks were stored in a freezer at −16°C for 1–10 days. Data represent the average of triplicate experiments with 10 test tubes per experiment

Medium composition: PDA, potato dextrose (2%) agar medium; PDTA, potato dextrose (1%) trehalose (1%) agar medium; PTA-2, potato trehalose (2%) agar medium; PTA-5, potato trehalose (5%) agar medium

Table 2. Effect of trehalose on the preservation of spawn of *L. edodes*: growth rate of mycelia

Days for freezing of spawn	0	1	3	10
PDA (control)	85.4 ± 7.52	25.7 ± 3.22	8.3 ± 0.75	0
PDTA	87.2 ± 6.75	31.0 ± 2.66	22.6 ± 3.12	11.1 ± 1.47
PTA-2	88.1 ± 9.64	43.3 ± 3.86	25.3 ± 1.96	7.4 ± 0.88
PTA-5	89.8 ± 7.73	35.9 ± 3.42	23.5 ± 2.17	3.2 ± 0.29

Data are mycelial dry weight (mg/50 ml) (average value ± standard deviation) ($n = 15$)
Mycelial disks were stored at −16°C for 1–10 days, inoculated in glucose peptone yeast extract (GPY) liquid medium, and incubated at 24°C
The dry weight of vegetative mycelia was measured 30 days after inoculation
Data represent the average of triplicate experiments with five polycarbonate vessels per experiment

Table 3. Effect of trehalose on the preservation of spawn of *L. edodes*: fruiting body formation

Days for freezing of spawn	0 (Control)	1	3	10
PDA (control)	71.4 ± 5.31	78.2 ± 7.68	0	0
PDTA	86.5 ± 8.87	69.3 ± 3.96	9.3 ± 0.72	0
PTA-2	78.7 ± 6.53	74.7 ± 8.88	7.3 ± 0.65	0
PTA-5	84.7 ± 9.15	73.1 ± 7.58	71.1 ± 8.47	0

Data are dry weight of fruiting bodies (mg/polycarbonate vessel) average value ± standard deviation) ($n = 15$)
The vegetative mycelia of *L. edodes* were grown on the above agar media. Then, the mycelial disks that had been stored at −16°C for 1–10 days were inoculated in glucose peptone yeast extract (GPY) liquid medium and incubated at 24°C. Induction of fruiting bodies was carried out by shifting the temperature to 15°C under about 100 ~ 200 lx light
The dry weight of fruiting bodies was measured at mature fruiting-body formation
Data represent the average of triplicate experiments with five polycarbonate vessels per experiment

cultivation of fungi in the PDTA medium before freezing. Compared to fruiting body yields, there was no difference for 1 day storage in the freezer. Although mycelia cultured in PTA-5 and then stored in the freezer for 3 days formed fruiting bodies satisfactorily, those in PDTA and PTA-2 medium formed only a few. Fruiting body formation was not observed when the mycelia were grown on PDA and then stored in the freezer for 3 days. On the other hand, 10 days of freezing resulted in poor mycelial growth (mycelia cultured in PDA medium had completely lost the ability to grow) and showed no fruiting body formation in any experiment.

For spawn storage of a basidiomycete, the subculture method was done by using a deep-freezer (−80°C), and a freezing method using liquid nitrogen (LN method, −160° to −196°C) (Jodon et al. 1982). The preservation method using a deep-freezer can be used for long-term storage; it is easy to manipulate and is used widely. However, *Amanita* does not survive by this method. Also, it is reported that with a term of storage, a survival ratio of this strain is described (Tan et al. 1991; Nakagiri 1997). A few forms of cultured mycelia, mycelial growth, and change of fruiting body formation by the LN freeze-dried method are good, but this method requires a large space and high cost (Maekawa 1997). For these reasons, the deep-freezers in the range of −80° to −100°C are used widely. The freezing method at a higher temperature (0° to −40°C) has also been used (Yokoyama 1992). This method has many problems as a long-term storage method because many strains do not survive.

To reveal the effect of trehalose on spawn storage of some edible mushroom fungi, we examined fungal stability against freezing using trehalose as the carbon source. Trehalose has been reported to be a kind of protectant for the cytoplastic membranes against dryness, freezing, and heat stress (Rudolph and Crowe 1985; Roser 1991). We studied the effect of trehalose in the temperature range of −10° to −19°C (average temperature, −16°C) under which conditions living cells were not stable. The survival rates of some edible mushroom fungi were increased by culturing in media which included trehalose as the growth substrate. The effect was more efficient in *P. ostreatus* and *L. edodes* than in *F. velutipes* in our experiments. The mycelia grown in trehalose media showed higher survival and growth rates and they formed more fruiting bodies after freezing than those grown in PDA. These results suggest that trehalose in the mycelia made fungi more stable against freezing.

Previously, we have reported the changes in the low molecular carbohydrates and trehalase activity during spawn storage of *L. edodes* mycelia (Terashita et al. 1997). Tan and Stalpers (1991) observed that the survival ratio of *C. phychomorbidus* and *S. commune* stored at −80°C was increased when these fungi were cultured in malt agar medium containing 5% trehalose. With regard to *C. psychomorbidus*, 0% survived when incubated in a medium not containing trehalose, but two of the nine colonies were viable in a medium containing trehalose. On the other hand, with regard to *S. commune*, one of the nine colonies survived even if incubated in a medium not containing trehalose, but all colonies incubated in a medium containing

trehalose were viable against freeze stress. The protective effects of trehalose for these organisms against cell damage were caused by residual moisture content in the medium. The residual moisture content of hyphal pellets that were incubated in medium supplemented with trehalose was consistently higher than those without trehalose. Hirasawa et al. (2001) examined the improvement of freeze tolerance for bakers' yeast by loading it with trehalose. When living cells were soaked in 0.5 M and 1.0 M trehalose solution, the maximum trehalose content in the cells reached almost 200–250 mg/g of dry cells. They described that loading the bakers' yeast with exogenous trehalose resulted in high freeze tolerance of cells, and this disaccharide, which usually causes freeze injury in the yeast, efficiently improved the survival ratio. However, we did not measure the amounts of exogenous trehalose-loaded vegetative mycelia when the mycelia were cultured in the medium including trehalose as the growth substrate. Investigations into the relationship between cellular trehalose content and the metabolic pathway for freeze tolerance in these mushroom fungi are now continuing. The results will be published elsewhere in the near future.

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