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EXPERIMENTAL ARTICLES

Biochemical Aspects of Basidiospore Maturation in *Agaricus bisporus* at Various Temperatures

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Abstract—Phosphatidylethanolamine is the main phospholipid of *Agaricus bisporus* basidiospores obtained under sterile conditions from young basidiomes with closed partial veils. Storing the basidiospores for five months at room temperature resulted in a complete loss of their germinating capacity. Conversely, storing them at a low temperature increased their germination rate by 15–20%. At both temperature levels, the phosphatidylcholine ratio significantly increased during storage to the level found in mature basidiospores. In addition, a drastic (8–10-fold) decrease in trehalose content occurred after two months of storage at room temperature. The trehalose content decreased only 1.5-fold at low temperatures. The involvement of trehalose and lipids in the retention of spore viability is discussed.

Key words: Agaricus bisporus, basidiospores, maturation, trehalose.

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The germination of *Agaricus bisporus* (Lange) Imbach basidiospores (BS) in the endogenous dormancy state is stimulated by the growing mycelium of some fungi [1], isovaleric acid [2], and heat shock [3]. However, under the effect of stimulators, the quantity of germinating BS does not exceed 10–15%. The point to be raised is why the rest of the basidiospores do not germinate. It is characteristic of research on basidial fungi that detached fruiting bodies with closed partial veils are used to obtain sterile BS. It is possible, therefore, that not all the spores thus obtained are mature. The process of BS formation by this method can be considered a model system that simulates the premature BS extrusion occurring in nature in response to unfavorable temperature conditions or dehydration.

It was reported in [4] that the germination of *A. bisporus* BS is enhanced by their storage at low temperatures. Conidia that cannot germinate are characterized by a peculiar lipid and carbohydrate composition [5]. Therefore, examining the lipid and carbohydrate composition of BS can help us resolve the question why only an insignificant fraction of them are viable. The trehalose content of dormant cells controls the spore-germination rate and their heat stability.

The goal of this work was to investigate the germinating capacity and the composition of lipids and cytosol carbohydrates of maturing BS at various temperatures.

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MATERIALS AND METHODS

This work used basidiospores from *Agaricus bisporus* 3718 fruiting bodies that were obtained from the Zarech'e Closed Joint-stock Company (Moscow Region). To obtain spore reprints, we sampled fruiting bodies (with a cap diameter of 35–40 mm) with closed partial veils. They were cleansed with cotton moistened with 50% and 70% ethanol. Thereupon, the covering was carefully removed with tweezers under sterile conditions and the stripe was cut at a point 1.0–1.5 cm below the cap edge. The *A. bisporus* fruiting bodies were then placed on sterile petri dishes and sterile cotton pads were spread over them. The dishes were loosely covered with lids. They were incubated at a temperature of 19–20°C. The spore reprints were obtained after two to three days. The fruiting bodies and the cotton pads were removed; the dishes were covered with lids and incubated for two days for drying. The spores produced from young basidiomes using the sterile method described above were termed BS-1. One half of the BS-1-containing petri dishes were kept at room temperature (20 ± 2°C), and the rest were placed in a refrigerator (2 ± 2°C). To obtain more mature BS, we used fruiting bodies with open partial veils. The BS thus obtained (termed BS-2) were not sterile.

The process of basidiome germination was monitored using an express method. A thin layer of melted agarized Goodwin medium [7] (2 ml) was applied on a slide. After the medium solidified, one drop of sterile spore suspension in water was evenly distributed over the slide surface with a glass stick. The slide was there-

Table 1. Lipid composition of *A. bisporus* basidiospores during storage at different temperatures

System	Storage conditions and time	Lipids, % of dry biomass	NL : GL :PL, % of total	Neutral lipids, % of total							Phospholipids, % of total		
				MG	DG	St	FFA	TAG	SE	HC	PC	PS	PEA
BS-1	–	19.4	77.5 : 13.8 : 8.7	–	3.8	4.9	2.5	86.7	2.0	–	6.6	–	93.4
BS-1	20°C, five months	16.7	73.6 : 13.3 : 13.1	–	2.1	7.2	–	76.9	6.6	7.2	63.1	–	36.9
BS-1	2°C, five months	21.1	77.8 : 12.5 : 9.7	1.1	3.2	7.0	–	78.0	9.5	1.1	32.2	–	67.8
BS-2	–	23.9	76.5 : 10.4 : 13.1	–	1.1	7.0	–	87.3	4.6	–	49.5	22.8	27.7

Note: NL, neutral lipids; GL, glycolipids; PL, phospholipids; DG, diacylglycerols; St, sterols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; HC, hydrocarbons; PC, phosphatidylcholine; PS, phosphatidylserine; PEA, phosphatidylethanolamine.

upon placed in a petri dish on filter paper moistened with 0.2 ml of water in order to make the atmosphere humid. The dishes were put into a desiccator with a cotton stopper and a 2-cm-thick water layer at the bottom. They were incubated at 24–26°C. The germinated basidiospores were counted after six days using an eyepiece grid mounted on the agar surface (at least 600 spores were counted in each study).

Lipids were extracted by the Folch method [8]. The separation of phospholipids (PL), glycolipids (GL), and neutral lipids (NL) was carried out on a column with L silica gel (100–160 mesh, Chemapol, Czech Republic) using solvents with different polarity degrees [9].

Thin-layer chromatography of lipids was performed on glass plates with KSKG silica gel (Lyaene Kalur, Estonia). NL were separated using the hexane–diethyl ether–acetic acid (85 : 15 : 1) solvent system. The hexane–diethyl ether–acetic acid (85 : 15 : 1) solvent system and the chloroform–methanol–acetic acid–water (25 : 15 : 4 : 2) solvent system were sequentially used to separate PL lipids. (50–100) μg were applied onto a plate. To develop the spots, the chromatograms were sprayed with 5% sulfuric acid in ethanol with subsequent heating to 180°C.

PL were identified using the individual markers phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (Sigma, United States) and the qualitative assays with ninhydrin (the test for amino groups) and Dragendorf reagent (the test for choline-containing phospholipids) [9]. Neutral lipids were identified using the following individual markers: mono-, di-, and triacylglycerols, free fatty acids, sterols (ergosterol), and hydrocarbons (Sigma, United States). Quantitative analysis of the chromatogram was carried out by scanning it with a Sorbphil-M densitometer (Sorppolymer, Russia).

The composition of fatty acid methyl esters was determined using a Model 3700 gas–liquid chromatograph (Russia) on a 2-m glass column packed with 17% diethyleneglycol succinate on Chromosorb W-AWDMSE-HP (80–100 mesh) as stationary phase; the temperature of the column was 180°C.

The carbohydrate composition of the cytosol of the basidiospores of the fungi was determined by extracting the sugars with boiling water for 20 min; the procedure was carried out four times. The spores were separated by centrifugation. Proteins were removed from the extract [10]. It was further purified on a column with a combination of Dowex-1 (acetate form) and Dowex 50W (H^+) resins. The carbohydrate composition was determined by gas–liquid chromatography, using trimethylsilyl sugar derivatives obtained from the lyophilized extract [11]. α-methyl-D-mannoside (Merck) was used as the internal standard. Chromatography was performed with a Model 3700 gas–liquid chromatograph (Russia) equipped with a flame ionization detector using a 2-m glass column with 5% SE-30 on 70–90 mesh Chromatone; the temperature was gradually raised from 130 to 270°C at a rate of 5–6 °C/min. Glucose, mannitol, arabitol, inositol, and trehalose (Merck) were employed as markers.

The statistical treatment of the results obtained was based on the sign test; the median value was determined (n = 3–4) [12].

RESULTS AND DISCUSSION

We compared the chemical composition of basidiospores (i) differing in their maturity degree; (ii) stored at 2 and 20°C for five months.

The comparison of freshly isolated basidiospores revealed that the lipid content of the more mature BS-2 was 20% higher than that of BS-1, while the NL : GL : PL ratio was virtually the same. NL accounted for 75–77% of the total lipids (Table 1). Triacylglycerols (86% of the total) are the main components of the neutral lipids. BS-2 are characterized by a significantly elevated content of sterol compounds and a lack of free fatty acids. The BS-2 phospholipids included phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylethanolamine (PEA) at a ratio of 2 : 1 : 1. In BS-1, PEA prevailed (93% of the total phospholipids).

The fatty acid composition of the neutral lipids of both BS types is virtually the same (Table 2). The main fatty acids are $C_{18:2}$ (68–69% of the total) and $C_{16:0}$ (14–16%). In contrast, the fatty acid composition of GL

Table 2. Fatty acid composition of the neutral lipids of *A. bisporus* basidiospores in relation to storage temperature

System	Storage conditions and time	Fatty acids, % of the total													DD*
		$C_{14:0}$	$C_{14:2}$	$C_{15:0}$	$C_{15:2}$	$C_{16:0}$	$C_{16:1}$	$C_{16:2}$	$C_{17:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:0}$	$C_{20:1}$	
BS-1	–	Traces*	–	Traces	–	14.3	4.6	–	Traces	3.1	8.6	69.4	–	–	1.52
BS-1	20°C, five months	2.4	–	–	–	15.0	–	–	1.5	4.1	6.8	65.5	–	–	1.38
BS-1	2°C, five months	7.0	4.2	4.2	1.9	10.7	–	3.2	3.2	11.1	7.1	48.4	–	8.5	1.27
BS-2	–	–	–	Traces	–	15.9	2.4	–	Traces	3.7	7.5	68.6	1.9	–	1.47

Note: DD, desaturation degree; Traces, content below 0.5%.

Table 3. Fatty acid composition of the glycolipids of *A. bisporus* basidiospores in relation to storage temperature

System	Storage conditions and time	Fatty acids, % of the total												DD
		$C_{14:0}$	$C_{14:1}$	$C_{15:0}$	$C_{15:1}$	$C_{16:0}$	$C_{16:1}$	$C_{16:2}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	X*	
BS-1	–	28.6	–	5.7	4.8	11.4	7.9	–	15.5	2.5	2.1	7.1	14.3	–	0.51
BS-1	20°C, five months	2.6	–	–	–	13.4	5.2	–	26.8	–	11.9	22.3	17.8	–	0.63
BS-1	2°C, five months	10.7	8.8	–	–	13.8	–	7.6	16.3	–	12.6	17.6	12.6	–	0.67
BS-2	–	–	–	–	–	23.7	–	6.6	6.6	–	9.9	18.4	9.9	25.0	0.76

Note: X, an unidentified fatty acid.

and PL is significantly different (Tables 3, 4). In BS-1 polar lipids, $C_{14:0}$ dominates, while both the α- and β isomers of linoleic acid are present. BS-2 is characterized by a lack of $C_{14:0}$, a high $C_{16:0}$ content (34% of the total), and the presence of only the α isomer of $C_{18:2}$. The desaturation degree of the fatty acids of PL and GL is 0.5–0.7 in both BS types, which is approximately two times lower than that of NL. The fact that saturated fatty acids dominate the membrane lipids of the zygospores of mucor fungi [13], another example of endogenously dormant cells, suggests that a low desaturation degree of membrane lipids is characteristic of endogenous dormancy. The conclusion to be drawn is that BS differing in their maturity degree are significantly different in the composition of their phospholipids and acyl chains and in their sterol content.

Storing BS-1 at 20°C for five months resulted in a complete loss of their germination capacity. In contrast, storing them at a low temperature increased the germination rate by 15–20% (Fig. 1). The data of Table 1 indicate that storing BS-1 at both temperatures caused changes in the NL and PL composition, so that it resembled that of BS-2: the percentage of PC and sterol compounds increased. A general trend is that C_{14}–C_{15} fatty acids appear in NL, PL, and GL during storage (Tables 2–4). The results obtained demonstrate that storage primarily affects the phospholipid composition.

Table 4. Fatty acid composition of the phospholipids of *A. bisporus* basidiospores in relation to storage temperature

System	Storage conditions and time	Fatty acids, % of the total																DD
		$C_{14:0}$	$C_{14:2}$	$C_{15:0}$	$C_{15:1}$	$C_{15:2}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$ α	$C_{18:2}$ β	$C_{20:0}$	X	$C_{21:1}$	
BS-1	–	38.9	–	5.4	6.0	–	8.8	6.4	9.6	3.6	2.4	2.4	3.6	12.6	–	–	–	0.51
BS-1	20°C, five months	7.4	–	5.2	2.6	–	7.9	–	11.3	3.9	5.2	–	15.2	10.5	30.6	–	–	0.58
BS-1	2°C, five months	5.4	2.1	–	1.8	–	4.2	–	6.0	–	4.8	1.8	13.6	13.6	–	16.9	29.6	0.92
BS-2	–	–	–	–	–	4.1	34.0	–	4.6	–	15.5	21.4	20.1	–	–			0.70

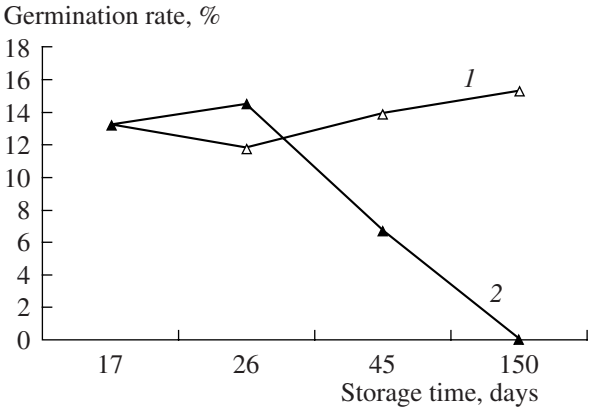

Fig. 1. Germinating capacity of *A. bisporus* basidiospores after storage at different temperatures: *1*, Storage at 2 ± 2°C; *2*, Storage at 20 ± 2°C.

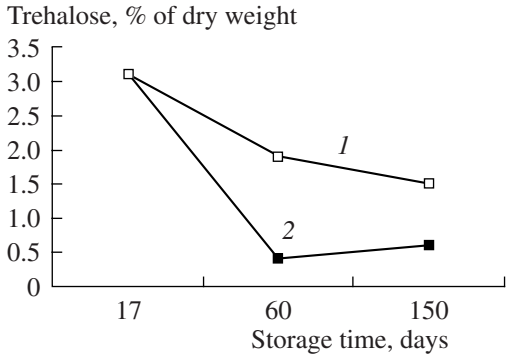

Fig. 2. Changes in trehalose content in basidiospores stored at different temperatures: *1*, Storage at 2 ± 2°C; *2*, Storage at 20 ± 2°C.

The PC/PEA ratio drastically increases, and a large quantity of long-chain fatty acids is formed. Thus, the changes in the phospholipid composition of BS-1 stored at room temperature and at a lower temperature are similar. However, a complete loss of germination capacity occurs at room temperature. In contrast, the germination capacity slightly increases (by 15–20%) at lower temperature. Since no significant differences between BS-1 stored at different temperatures were detected, we cannot attribute this loss of viability to the peculiarities of their lipid composition. Moreover, the lipid content of BS-1 decreases only by 15% after five months of storage at 20°C, in contrast to the data obtained at 2°C.

The carbohydrate composition demonstrates considerable changes during storage. The carbohydrate content reaches 12%, and mannitol (74–75% of the total) and trehalose (24–25%) prevail. The mannitol content decreases 3–4-fold, regardless of the temperature. The trehalose content changes are different at different temperatures. The trehalose content decreases 7–8-fold at 20°C and only 1.5-fold at 2°C (Fig. 2). The gradual decrease revealed by us in carbohydrate content during storage suggests that BS cannot maintain their carbohydrate level, in contrast to exogenously dormant *A. niger* conidia [14].

In nature, premature extrusion of BS at unfavorable temperature and humidity levels is a sufficiently widespread phenomenon. Therefore, the maturation capacity of BS is an adaptation enabling species preservation under deleterious conditions. In this study, we revealed for the first time the biochemical peculiarities of the process of basidiospore maturation. Immature fruiting bodies with closed coverings form basidiospores that are morphologically undistinguishable from mature basidiospores. However, their chemical composition is markedly different. BS-1 should be regarded as vegetative rather than dormant cells in terms of their phospholipid composition [13]. This study demonstrates that low temperatures promote the survival of BS. BS ripening is accompanied by replacement of PEA by PC, which is characteristic of dormant cells [15]. In addition, no drastic decrease in trehalose content occurs at a low temperature. Trehalose is a protective carbohydrate; its content controls the spore germination rate and thermostability [6]. This may account for the fact that fruition in basidiomycete fungi occurs in the fall, shortly before the low temperature period.

The research carried out by us reveals that (i) the germinating capacity of basidiospores varies depending on their trehalose level and (ii) the carbohydrate content gradually decreases during the storage period. These data suggest that the original low germination level of BS-1 (15% or less) is due to the low trehalose level in the other 85% of the basidiospores involved.

REFERENCES

1. Lösel, D.M., The Stimulation of Spore Germination in *Agaricus bisporus* by Living Mycelium, *Ann. Bot. N.S.*, 1964, vol. 28, pp. 541–554.
2. Lösel, D.M., The Stimulation of Spore Germination in *Agaricus bisporus* by Organic Acids, *Ann. Bot. N.S.*, vol. 31, pp. 417–425.
3. Feofilova, E.P., Tereshina, V.M., Garibova, L.V., Zav'yalova, L.A., Memorskaya, A.S., and Maryshova, N.S., Germination of Basidiospores of *Agaricus bisporus, Prikl. Biokhimiya I Mikrobiologiya*, 2004, vol. 40, no. 2, pp. 220–226 [*Appl. Biochem. Microbiol.* (Engl. Transl.), vol. 40, no. 2, pp. 186–191].
4. Safrai, A.I., Biological Characteristics of Certain *Agaricus* FR. Emend. Karst. Species, *Cand. Sci. (Biol.) Dissertation*, Moscow: Mosk. Gos. Univ., 1977.
5. Morozova, E.V., Kozlov, V.P., Tereshina, V.M., Memorskaya, A.S., and Feofilova, E.P., Changes in Lipid Composition and Carbohydrate Composition of *Aspergillus niger* Conidia during Germination, *Prikl. Biokhim. Mikrobiol.*, 2002, vol. 38, no. 2, pp. 149–154 [*Appl. Biochem. Microbiol.* (Engl. Transl.), vol. 38, no. 2, pp. 129–133].

6. Tereshina, V.M., Resting Cells and Adaptation of Mycelial Fungi to Heat Shock, *Doctoral (Biol.) Dissertation*, Moscow: INMI RAN, 2006.
7. Garton, C.U., Goodwin, T.W., and Lijinsky, V., Studies in Carotenogenesis of *Phycomyces blakesleeanus, Biochem. J.*, 1961, vol. 48, pp. 154–163.
8. Folch, J., Lees, M., and Sloane-Stanley, G.H.S., A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem.*, 1957, vol. 226, pp. 497–529.
9. Keits, M., *Techniques of Lipidology: Isolation, Analysis, and Identification of Lipids*, Amsterdam: Elsevier, 1972 [Rus. Transl. Moscow: Mir, 1975].
10. Somogyi, H., Determination of Blood Sugar, *J. Biol. Chem.*, 1945, vol. 160, p. 69.
11. Brobst, K.M., Gas-Liquid Chromatography of Trimethylsililic Sugar Derivatives, *Metods of Carbohydrate Research*, Khorlin, A.Ya, Ed., Moscow: Mir, 1975.
12. Ashmarin, I.P. and Vorob'ev, A.A., *Statisticheskie metody v mikrobiologicheskikh issledovaniyakh* (Statistical Methods in Microbiological Research), Leningrad: Gos. Izd-vo med. literatury, 1962.
13. Tereshina, V.M., Memorskaya, A.S., Kochkina, G.A., and Feofilova, E.P., Dormant Cells in the Developmental Cycle of *Blakeslea trispora:* Distinct Patterns of the Lipid and Carbohydrate Composition, *Mikrobiologiya*, 2002, vol. 71, no. 6, pp. 794–800 [*Microbiology* (Engl. Transl., vol. 71, no. 6, pp. 684–689.
14. Tereshina, V.M., Kovtunenko, A.V., Memorskaya, A.S., and Feofilova, E.P., Effect of Carbohydrate Composition of the Cytosol of *Aspergillus niger* Conidia on Their Viability during Storage, *Prikl. Biokhim. Mikrobiol.*, 2004, vol. 40, no. 5, pp. 527–532 [*Appl. Biochem. Microbiol.* (Engl. Transl.), vol. 40, no. 5, pp. 454–459].
15. Feofilova, E.P., Deceleration of Vital Activity as a Universal Biochemical Mechanism Ensuring Adaptation of Microorganisms to Stress Factors: A Review, *Prikl. Biokhim. Mikrobiol.*, 2003, vol. 39, no. 1, pp. 5–24 [*Appl. Biochem. Microbiol.* (Engl. Transl.), vol. 39, no. 1, pp. 1–18].