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EXPERIMENTAL ARTICLES

A New Function of Trehalose and the Peculiarities of Lipid Formation in Mycelial Fungi

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Abstract—This work deals with lipid formation in ascomycete fungi and the effect of preservatives on them. A new biological function of trehalose was revealed, and of particular interest was the fact that the effect of this disaccharide depended on its concentration in the growth medium. In the presence of a preservative such as potassium sorbate (PS), low trehalose concentrations suppressed the growth of mycelial fungi contaminating hard cheeses and contributed to the prolongation of the preservative's effect. A tenfold increase in trehalose concentration in the medium, conversely, resulted in a drastic increase in growth activity and removed the PS effect. Therefore, trehalose can function as an inhibitor or a stimulator of growth processes, depending on its concentration. It was established that the secondary growth of *Penicillium* fungi during their ontogeny is accompanied by consumption of accumulated reserve lipids. In contrast, this phenomenon does not occur in mucorous fungi, and this probably accounts for the fact that *Mucorales* representatives can accumulate significant triacylglyceride amounts during the idiophase.

Key words: mycelial fungi, trehalose, lipids, potassium sorbate preservative, secondary growth.

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Trehalose is a carbohydrate belonging to the group of nonreducing disaccharides. Two D-glucose residues are linked by an α,α-glycoside bond in native trehalose. Owing to its special role in anabiosis, this disaccharide has always received much attention of biologists and chemists. By the beginning of the 20th century, it was established that trehalose plays a polyfunctional role in a large number of living organisms. It has been revealed that trehalose is an energy source and a reserve carbohydrate. Under stress, it stabilizes proteins and membrane lipids and protects them against degradation. It prevents the destructive effect of free radicals on biological structures, operates as a regulator of growth and development in plants, and is a glycolipid component in corynebacteria [1].

One of the goals of this work was to investigate the influence of trehalose on mycelial fungi under the extraordinary conditions created by the preservative potassium sorbate (PS). The studies were conducted with ascomycete fungi (*Penicillium*) that are the most active colonizers of hard cheeses in agribusiness plants. The other goal was to elucidate the relationship between growth activity and lipid formation in these fungi and to investigate their difference in this respect from mucorous fungi that also spoil hard cheeses.

Attaining these goals promoted the implementation of the results obtained, i.e., achieving a prolonged effect of PS, which is the most widely used preservative in food industry.

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MATERIALS AND METHODS

The studies were conducted with four cheese-colonizing representatives of ascomycete fungi isolated in agribusiness plants: *Penicillium chrysogenum* [2], *P. variabile* [3], *P. roqueforti* [4], and *P. aurantiogriseus* [5], as well as with *Cunninghamella japonica* BKMF 1204 (–) [6], which belongs to the mycelial fungi of the order *Mucorales*.

Ascomycete mycelial fungi were cultivated on the Blumenthal–Rosemann medium, the composition of which was as follows (%): sucrose, 3.0; $(NH_4)_2SO_4$, 1.0, KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1; KCl, 0.05; yeast extract, 0.03; $FeSO_4 \cdot 7\,H_2O$, 0.001; and $ZnSO_4 \cdot 7H_2O$, 0.005. The cultivation was carried out in 250-ml flasks with 50 ml of medium on a rotary shaker (220 rpm) at a temperature of 27°C. *C. japonica* was cultivated on Goodwin medium [7] and on a ammonium nitrate-containing medium [8]. In studies on the effect of the preservative potassium sorbate on ascomycete fungi, whey was used as the growth medium [9]. Lipids were extracted according to [10].

The composition of the neutral lipid (NL) and polar lipid (PL) fractions was analyzed by ascending thin-layer chromatography on plates with a fixed silica gel layer (Merck, Germany). NLs were separated using the hexane–diethyl ether–acetic acid (85 : 15 : 1) or the petroleum ether–diethyl ether–acetic acid (80 : 20 : 2) solvent systems [10]. PLs were separated by two-dimensional TCL, consecutively running the chloroform–methanol–water (65 : 25 : 4)

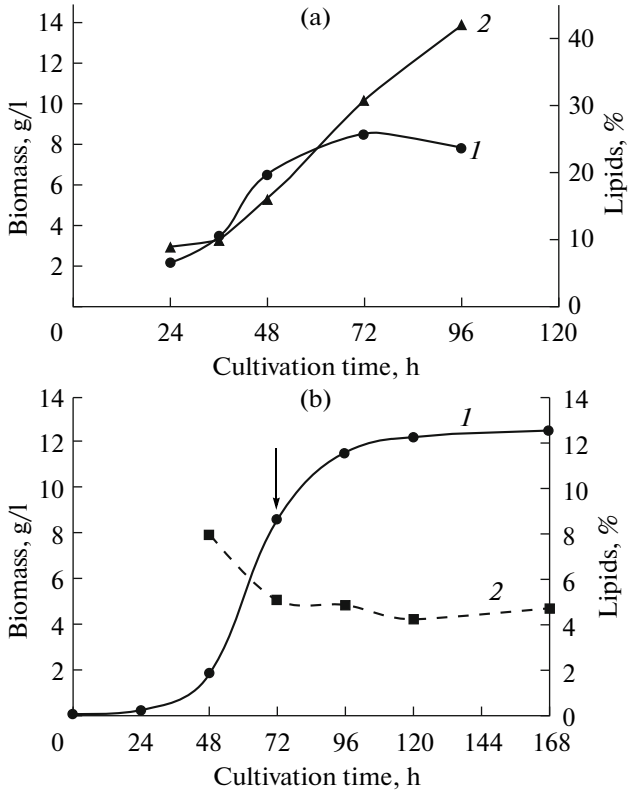

Fig. 1. Changes in biomass yield (*1*) and lipid content (*2*) during the growth of *C. japonica* (a) and *P. roqueforti* (b). Arrow, onset of secondary growth.

system in one direction and the chloroform–acetone–methanol–acetic acid–water (50 : 20 : 10 : 10 : 5) system in the other [11]. In addition, 70–120 μg of lipids were applied on a plate (Merck, Germany).

The fractions obtained were identified using standard lipid preparations and specific qualitative tests for individual functional groups [10]. For this purpose, we used the Vaskovsky and the Dragendorff reagents, ninhydrin and α-naphthol solutions, and a mixture of sulfuric and acetic acid. Phosphomolybdic acid was used as the universal developer.

Quantitative analysis of individual lipids was carried out using the Dens software package (Lenchrom, Russia).

The statistical treatment of the results was performed by the median (Me) method with $n = 2–3$ [12].

RESULTS AND DISCUSSION

Fungal species spoiling food items such as hard cheeses were investigated earlier [9]. The most frequent food-spoiling organisms are ascomycete fungi while mucorous fungi are significantly less widespread. In our subsequent studies, we demonstrated that potassium sorbate (PS), the most widely used preservative, inhibits the growth of mycelial fungi by affecting their lipid metabolism [13, 14]. The preservative lowers the content of linoleic acid, which is the most important component of the stress protection system of living organisms.

We also established that growth processes in fungi such as *P. roqueforti* resumed 7–8 days after the addition of PS and were carried out even more actively than in the control system. Since PS is widely used in the food industry as a preservative that prevents molds from growing on cheese, research aimed at prolonging the PS effect is of paramount importance.

Taking into account the fact that PS influences the lipid composition of fungal mycelium as a stress factor, we decided to conduct preliminary studies in order to investigate its effect on the lipid composition of contaminating fungi such as *P. roqueforti*. In this fungus, PS is degraded by enzymes [15], resulting in growth resumption. Of particular interest is the fact that secondary growth of *Penicillium* proceeds under the influence of an additional stress factor, incipient depletion of the nutrient medium, i.e., starvation. Therefore, we considered it necessary to investigate the effect of nutrient depletion (starvation) on the lipid composition of cheese-contaminating fungi, including mucorous and ascomycete (*Penicillium*) species. The former are known to be more sensitive to the PS effect [14].

Studies on lipid formation in the mucorous fungus *C. japonica* revealed the following trends (exemplified by the results obtained on the Goodwin medium and shown in Figs. 1a and 2a).

(i) Biomass accumulation proceeded until day 4 and ceased by day 5 of cultivation. The biomass yield on the Goodwin and ammonium nitrate-containing medium was approximately 8–10 and 15 g/l (not shown), respectively. The content of the lipids accumulated in the fungal biomass by the end of the growth period was ~40–50 and ~45%, respectively.

(ii) The total lipid amount increased throughout the whole growth period and reached the maximum value by the end of the idiophase.

(iii) The neutral lipid content also increased in the course of the growth period and reached the maximum value by the end of the idiophase. The reverse trend was observed for the polar lipids.

(iv) At transition to the stage of active growth, phosphatidylethanolamine (PEA) was the predominant phospholipid, whereas growth deceleration was accompanied by a drastic increase in phosphatidylcholine (PC) content.

Similar experiments with ascomycete fungi revealed different trends in terms of their growth and lipid formation (exemplified by the results obtained by cultivating *P. roqueforti* on the Blumenthal–Roseman medium and shown in Figs. 1b and 2b):

(i) Biomass accumulation increased during the growth of the fungi; the PC content on day 2 was higher than on day 4 of cultivation.

(ii) On day 4, a decrease in the total lipid content occurs; the triacylglyceride (TAG) amount decreased, while the polar lipid amount increased.

(iii) By day 4 of cultivation, the PEA level increased drastically and the PC level decreased; the phosphatidic acid (PA) content increased.

Microscopic examination revealed that thin mycelium was formed de novo by *Penicillium* fungi after 50–60 h of growth. The mycelium had a very thin cell wall and virtually lacked lipid inclusions (the secondary growth phenomenon). It coexisted with thick-walled hyphae that were 1.5–2.0 times larger in diameter and contained lipid granules. Subsequently (by day 4), such hyphae lost their intracellular content and lipid inclusions disappeared.

Such phenomena did not occur during the cultivation of the mucorous fungus. Lipids did not disappear upon growth cessation. Microscopic examination demonstrated the presence of specific structures: by this time, the mycelium contained hyphae with thickened cell walls and ampoulelike bulges with large lipid bodies.

These data gave grounds for the suggestion that, in contrast to mucorous fungi, *Penicillium* fungi do not assume a dormant state upon nutrient depletion. A new mycelium generation is formed, previously accumulated lipids are consumed, and secondary growth of the biomass occurs. Interestingly, these trends in lipid formation in the representatives of two different systematic groups are quite consistent with the data on their lipid composition. In our studies generalized in earlier work [16], it was established by the example of mucorous fungi that the active growth period corresponded to a particular lipid composition characterized by predominance of PEA and a significantly lower PC content. The subsequent growth deceleration and lipid accumulation resulted in a substantial change in the ratio of these bulk lipids associated with a considerable PC accumulation. Leaving out of consideration the capacity of *Penicillium* fungi for secondary growth, we might assume that the above trend is not valid for this system. However, it is evident from the data of Figs. 1 and 2 that the onset of secondary growth coincides with an increase in PEA content in the case of *Penicillium* fungi. Accordingly, our conclusion concerning the dependence of membrane lipid composition on growth activity [16] also applies to other systematic groups of fungi, including ascomycetes, although they have their peculiarities. As our further studies with PS revealed, it is the fungi's capacity for secondary growth that provided evidence for a new role of trehalose and the dependence of its effect on trehalose concentration in the medium.

Our research on mycelial fungi that spoil food at agribusiness plants revealed that PS does not exert a long-term effect. On day 5 after the addition of the preservative, its degradation by enzymes of ascomycete fungi occurs [15] and the growth of secondary mycelium starts. This growth is more intense than in the control system (Fig. 3). The control biomass yield reaches 8–9 g/l by this time and remains virtually unchanged during the subsequent cultivation period. However, if 0.1% trehalose is added to the cultivation medium in combination with PS, the growth of the fungus is delayed until day 7–8. Subsequently, growth processes start; eventually, the result slightly exceeds than in the control system.

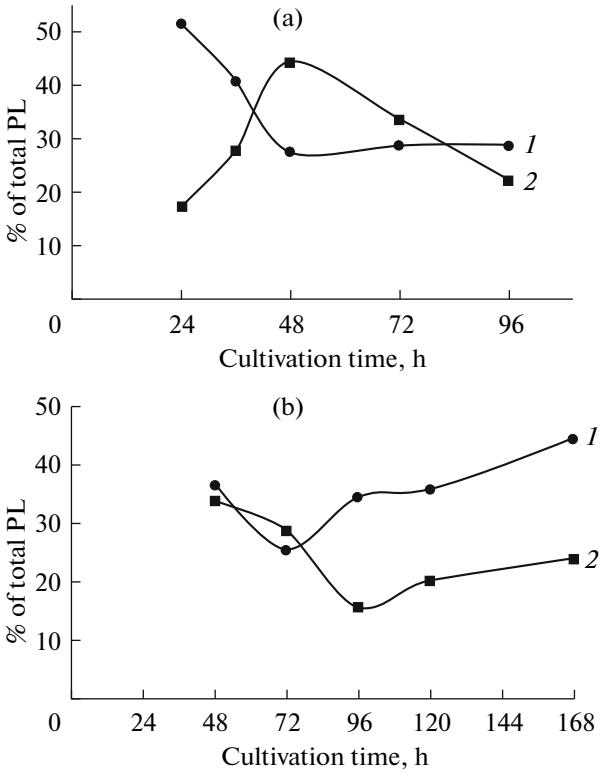

Fig. 2. Changes in the contents of the individual PEA (*1*) and PC (*2*) phospholipid fractions during the growth of *C. japonica* (a) and *P. roqueforti* (b).

Even more fascinating data on the trehalose effect in the presence of PS are obtained if a much (ten times) larger trehalose concentration of 1% is added along with the preservative. In this system, PS works as a preservative for a significantly shorter time, and on day 7, the biomass already becomes half as abundant as in the control system.

It was shown for the first time, therefore, that trehalose, which is dubbed the "dormancy sugar," inhibits the effect of the preservative PS and stimulates the growth of cheese-contaminating mycelial fungi. Of particular interest is the fact that the growth-stimulating effect of trehalose depends on its concentration and manifests itself at a sufficiently high content of this disaccharide. If the trehalose concentration in the medium does not exceed 0.1–0.5%, this protective carbohydrate exerts an inhibitory influence on the growth of fungi and prolongs the effect of the preservative.

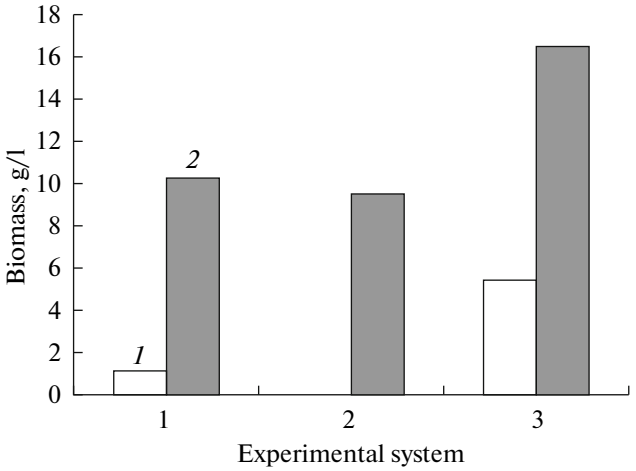

Fig. 3. Influence of PS and trehalose (Th) on *P. roqueforti* growth (*1*, day 7; *2*, day 10): 0.02% PS (1), 0.02% PS + 0.1% Th (2), 0.02% PS + 1% Th (3).

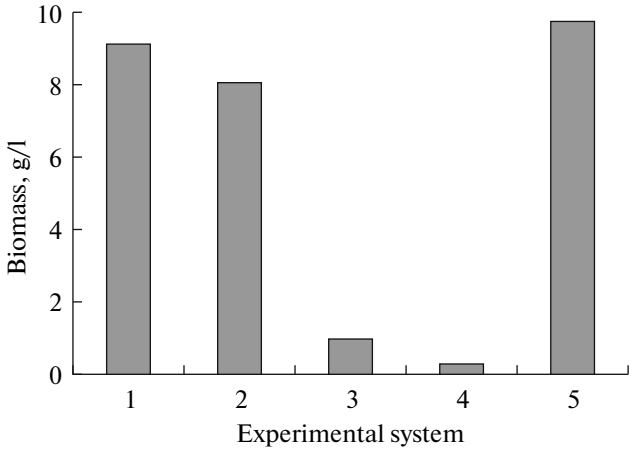

Fig. 4. PS effect and its prolongation by additional substances (day 10): control (1), 0.02% PS (2), 0.02% PS + 1% CH_3COONa + 1% NaCl (3), 0.02% PS + 1% CH_3COONa + 1% NaCl + 0.1% Th (4), 0.02% PS + 1% CH_3COONa + 1% NaCl + 1% Th (5).

This pattern of trehalose effect persists if the PS effect was tested under different conditions. We established [17] that the inhibitory effect of PS on *Penicillium* growth can be prolonged by adding other preservatives such as sodium acetate and common salt at a concentration of 1% (Fig. 4). If the above medium composition was supplemented with 0.1% trehalose, the prolongation of the PS effect became more significant, as noted above. If the trehalose concentration was increased to 1%, this resulted in a considerable intensification of growth processes, in line with the trends emphasized above.

Thus, two new facts related to the peculiar mode of action of trehalose were established in this work. Currently, it is assumed that trehalose accumulates in spores (dormant cells) and its utilization as a carbon source starts while the cell is in a dormant state. Initially, the enzyme trehalase starts functioning. It cleaves trehalose into two glucose molecules. This work demonstrates that trehalose functions as an agent removing the preservative's effect and enabling the fungus to grow. However, this peculiar function of trehalose requires its sufficiently high content in the cultivation medium (over 1%). If the concentration is ten times lower, trehalose behaves as a growth process inhibitor, i.e., performs the familiar function of a natural preservative in the presence of a stress factor. In this context, the data that glucose and lactose fail to produce a similar effect are of special interest.

Currently, it is assumed that nonreducing sugars, such as trehalose and sucrose, are involved in the operation of the cell communication system (glycocode) and their metabolism is linked with the cell signaling machinery [18]. Research on the effect of sucrose on the growth of cheese-spoiling fungi in the presence of PS was of paramount importance in view of the unique properties of nonreducing sugars and a certain similarity between the biological functions of trehalose and sucrose. It has been established that sucrose activates fungal growth at a concentration of 1%. In contrast to trehalose, low sucrose concentrations (0.1%) added in combination with the preservative failed to prolong its effect on fungal growth. Presumably, the extraordinary effect of trehalose is due to the unique physical properties of this molecule, such as its high hydrophilicity, chemical stability, and lack of internal hydrogen bonds [19].

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REFERENCES

1. Elbein, A.D., Pan, Y.T., Pastuszak, I., and Carroll, D., New Insights on Trehalose: a Multifunctional Molecule, *Glycobiology*, 2003, vol. 13, no. 4, pp. 17R–27R.
2. Thom, C., Cultural Studies of Species of *Penicillium, Bull. Bureau Animal Industry,* United States Dept. Agriculture, 1910, vol. 118, pp. 1–109.
3. Sopp, O.J., Monographie der Pilzgruppe *Penicillium* mit besonderer Berücksichtigung der in Norwegen gefundenen Arten, *Skirifter udgivne as Videnskabs-Selskabet i Christiania. Mathematisk-Naturvidenskabelig Klasse*, 1912, vol. 11, pp. 1–208.
4. Thom, C., Fungi in Cheese Ripening: Camembert and Roquefort, *Bull. Bureau Animal Industry*, United States Dept. Agriculture, 1906, vol. 82, pp. 1–39.
5. Dierckx, R.P., Un essai de revision du genre *Penicillium* Link, *Annals de Societe de Sciences Bruxelles*, 1901, vol. 25, pp. 83–89.
6. Ito, S., *Mycological Flora of Japan, vol. 1. Phycomycetes*, Tokyo: Yokendo, 1936.

7. Garton, G.A., Goodwin, T.W., and Lijinsky, W., Studies in Carotenogenesis. 1. General Conditions Governing β-Carotene Synthesis by the Fungus *Phycomyces blakesleeanus* Burgeff, *Biochem. J.,* 1951, vol. 48, no. 2, pp. 154–163.

8. Sergeeva, Ya.E., Galanina, L.A., Andriyanova, D.A., and Feofilova, E.P., Lipids of Filamentous Fungi as a Material for Producing Biodiesel Fuel, *Prikl. Biokhim. Mikrobiol.*, 2008, vol. 44, no. 5, pp. 576–581 [*Appl. Biochem. Microbiol.* (Engl. Transl.), vol. 44, no. 5, pp. 523–527].

9. Feofilova, E.P., Kuznetsova, L.S., Sergeeva, Ya.E., and Galanina, L.A., Species Composition of Food-Spoiling Mycelial Fungi, *Mikrobiologiya,* 2009, vol. 78, no. 1, pp. 128–133 [*Microbiology* (Engl. Transl.), vol. 78, no. 1, pp. 112–116].

10. Keits, M., *Techniques of Lipidology: Isolation, Analysis, and Identification of Lipids*, Amsterdam: Elsevier, 1972 [Russ. Transl. Moscow: Mir, 1975].

11. Benning, C., Huang, Zh.-K., and Gage, D.A., Accumulation of a Novel Glycolipid and a Betaine Lipid in Cell of *Rhodobacter sphaeroides* Grown under Phosphate Limitation, *Arch. Biochem. Biophys.*, 1995, vol. 317, pp. 103–111.

12. Ashmarin, I.P. and Vorob'ev, A.A., *Statisticheskie metody v mikrobiologicheskikh issledovaniyakh* (Statistical Techniques in Microbiological Research), Leningrad: Meditsinskaya literatura, 1962.

13. Ovcharova, T.P., *Primenenie sorbinovoi kisloty v pishchevoi promyshlennosti* (Sorbic Acid in Food Industry), Moscow: Pishchevaya promyshlennost', 1966.

14. Sergeeva, Ya.E., Galanina, L.A., Kochkina, G.A., and Feofilova, E.P., The Effect of the Preservative Sorbic Acid on the Lipid Composition of the Ascomycete Fungus *Penicillium roqueforti* Thom, *Mikrobiologiya*, 2009, vol. 78, no. 5, pp. 695–701 [*Microbiology* (Engl. Transl.), vol. 78, no. 5, pp. 630–635].

15. Stopforth, J.D., Sofas, J.N., and Busta, F.F., Sorbic Acid and Sorbates, in *Antimicrobials in Food, 3rd ed.,* Davidson, P.M., Sofos, J.N., and Branen, A.L., Eds., CRC Press Taylor & Francis Group, 2005, pp. 49–90.

16. 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].

17. Feofilova, E.P., Galanina, L.A., Sergeeva, Ya.E., Memorskaya, A.S., Snezhko, A.G., and Mysyakina, I.S., RF Patent No. 2009100136 (2009).

18. Paul, M.J., Primavesi, L.F., Jhurreea, D., and Zhang, Y., Trehalose Metabolism and Signaling, *Ann. Rev. Plant Biol.*, 2008, vol. 59, pp. 417–441.

19. Aranda, J.S., Salgado, E., and Taillandier, P., Trehalose Accumulation in *Saccharomyces cerevisiae* Cells: Experimental Data and Structural Modeling, *Biochem. Eng. J.*, 2004, vol. 17, no. 2, pp. 129–140.