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EXPERIMENTAL ARTICLES

Heat Shock Response in the Thermophilic Fungus *Rhizomucor miehei*

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Abstract—Changes in the composition of the membrane lipids and cytosol carbohydrates of the thermophilic fungus *Rhizomucor miehei* in response to heat shock were studied. Under optimal conditions (41–43°C), high trehalose content (8–11%) was found at all stages of growth of submerged culture. Heat shock (51–53°C) for 1 h did not result in enhanced trehalose synthesis, while increase in shock duration to 3 h resulted in a significant increase in trehalose content. The share of sterols and phosphatidic acids in the membrane lipids increased, while the share of phosphatidylcholines and phosphatidylethanolamines decreased. These processes resulted in increased content of non-bilayer lipids, while the unsaturation degree of the fatty acids of the major phospholipids did not decrease. Comparison of resistance to lethal heat shock in the control and experimental variants of *R. miehei* revealed that this thermophilic fungus exhibited no acquired heat resistance.

Keywords: *Rhizomucor miehei*, heat shock, heat resistance, membrane lipids, phosphatidic acids, trehalose

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The response of microorganisms to heat exposures is being widely studied on the models of mesophilic fungi for which three membrane defense hypotheses were proposed. The first homeoviscous adaptation hypothesis [1] postulates the maintenance of certain membrane viscosity by changing the unsaturation degree of phospholipid acyl chains. The second homeophasic adaptation hypothesis [2] focuses attention on the maintenance of balance between bilayer (cylinder-shaped) and nonbilayer (cone-shaped) lipid molecules. However, both hypotheses do not take into account the differences between heat effects in the tolerance zone, where only reduction in the growth rate is observed, and the heat shock (HS), which results in the cessation of the growth processes. Comparison between biochemical changes in fungal cells under these two types of exposure showed their fundamental difference, which allowed us the third stabilization hypothesis to be proposed [3], according to which the maintenance of membrane structural organization under HS conditions is carried out with the involvement of the compounds stabilizing the lipid bilayer, namely, trehalose, sterols, glycolipids, etc. An activation of the defense system in HS that includes the synthesis of heat shock proteins, trehalose accumulation, a change in the state of water in cell compartments and in the cell composition, etc. [4] results in the acquisition of a new property—heat resistance. However, the evidences of stress response in thermophilic fungi are scanty and contradictory.

The growth optimum of thermophilic fungi is 40–45°C, which is the cause of a heat shock in thermophilic fungi. When we studied thermophilic fungi, the main attention was focused on the comparison between the composition of membrane lipids of thermophilic and mesophilic fungi [5, 6]. It was established that thermophils synthesize more saturated lipids than mesophils and psychrophils. A large number of phosphatidic acids were found in the representative of thermophils *Thermomyces lanuginosus* (syn. *Humicola grisea* var. *thermoidea* [7], which allowed us to suggest a special role of this compound in the life of the fungi at elevated temperatures.

What should be noted is an attempt to consider the problem of thermophilia from the standpoint of adaptation of mesophilic fungi to heat shock [8–10]. The authors suggested that at elevated temperatures thermophils depended for life on the mechanisms, which are involved in heat shock response in mesophils. This suggestion is based on the fact that a considerable amount of trehalose (3.0–3.5% of dry mass), comparable to that in mesophilic fungi under HS conditions, was revealed in the thermophilic fungus *Myceliophthora thermophila* during the active growth phase under the optimal conditions. Growing the fungus at

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different temperatures resulted in different phospholipid compositions: at optimal temperatures, phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylethanolamide (PE) predominated; at suboptimal temperatures, PC and PE. In addition, the presence of adaptation Δ^{15}-desaturase was shown in the fungus, which refuted the hypothesis that life at low temperatures was impossible for thermophils due to the absence of this enzyme that would increase the unsaturation degree of phospholipid fatty acids when the temperature decreased.

The comparative study of HS response in two closely related species, the mesophilic fungus *Chaetomium brasiliense* and the thermophilic fungus *Chaetomium thermophile* var. *thermophile*, is of interest [11]. It was shown that the fatty acids of the total lipids of the thermophilic fungus are more saturated than in the mesophilic fungus; it was established that, under optimal conditions, both fungi synthesized comparable amounts of trehalose and increased its amount in response to HS. But note that the thermophilic fungus forms less trehalose than the mesophilic one, which led the authors to the conclusion that no relationship was present between trehalose and thermophilia. The absence of comparison with HS response of the control variant, the vagueness of the temperature characteristics of the fungus growth, and the analysis of the fatty acid composition of the total (not membrane) lipids were drawbacks of this interesting study. The presence of acquired heat resistance was shown in another thermophilic fungus: *Thermomyces lanuginosus*; however, the studies were conducted with conidial sprouts (4 h), but not with mycelium, and the amount of trehalose was not determined either [12]. As a result, scarce data on the formation of HS response of thermophilic fungi do not allow us to understand whether the phenomenon of acquired heat resistance is inherent in them and what mechanisms they use for adaptation to HS.

The aim of this study was to study the heat resistance and the membrane lipid and the cytosol carbohydrate composition in the thermophilic fungus *R. miehei* under HS conditions.

MATERIALS AND METHODS

The subject of study and cultivation conditions. The thermophilic fungus *Rhizomucor miehei* VKM F-1365 (*Zygomycetes, Mucorales, Mucoraceae*) was studied. The culture was grown on slant wort agar at an optimal temperature of 42–43°C for five to six days, kept at room temperature, and passaged once a month. The spore suspension, which was introduced into a liquid medium and adjusted to an end concentration of 5×10^5–10^6 spores/mL medium, was used for inoculation.

The surface 24-h culture of *R. miehei* grown on wort agar at 41–42°C was used as inoculum to investigate the temperature characteristics of the fungus growth. The circles of this culture with a diameter of about 10 mm were placed at the center of petri dishes with wort agar 7°B and grown in a thermostat for 24 h at nine different temperatures varying between 20 and 55°C. To determine linear growth, we measured the colony diameter in two mutually perpendicular directions.

The submerged culture of the fungi was grown in 250-mL flasks with 50 mL of Goodwin medium [13] using the KE-12-250T electromagnetic shaker at 150 rpm at an optimal temperature of 42–43°C for 24 h. In order to study thermal influences, the 24-h culture was transferred to the 52–53°C conditions maintaining the same aeration conditions and allowed to grow for 1 and 3 h (variants HS-1 and HS-3, respectively). The control variants (C-1 and C-3) were cultivated under the temperature optimum conditions for the same time.

In order to determine the lethal heat shock temperature, the aliquots (4 mL) of 24-h submerged culture were placed in test tubes and heated at 55–72°C at a pace of 3°C for 20 min; petri dishes with agarized wort (42–43°C) were then inoculated with the culture without its being cooled and grown for 48 h, with the viability being assessed by the presence of colony growth.

In order to reveal acquired thermotolerance, the experimental variants C-1, C-3 and HS-1, HS-3 were heated at a lethal heat shock temperature (69°C, 20 min), subsequently inoculating the petri dish with agarized wort with the culture and growing it for 48 h, after which the fungus viability was assessed.

Analysis of the membrane lipids. The fungal mycelium was filtered through Capron and washed with distilled water of the corresponding temperature; the raw biomass sample weight in isopropanol was homogenized, after which lipid extraction was continued for 30 min at 70°C and the supernatant fluid was decanted [14]. Further, the residue was extracted with the isopropanol : chloroform mixture (1 : 1) twice under the same conditions. The pooled extract was evaporated in a rotary evaporator; the residue was dissolved in 3 mL of the chloroform : methanol mixture (1 : 1) to which 4 mL of 2.5% sodium chloride was added to remove water-soluble substances. After separating the mixture, the chloroform layer was sampled and dried with anhydrous sodium sulfate, evaporated on the rotary evaporator and dried in a vacuum until the formation of a constant mass. The residue obtained was dissolved in the chloroform : methanol mixture (1 : 1) and kept at –21°C.

The composition of neutral lipids (NL) was analyzed with ascending TLC on glass plates with silica gel 60 (10 × 10 cm) (Merck, Germany). The solvent system hexane : sulfuric ether : acetic acid (85 : 15 : 1) was used for NL separation [15]. Phospho- and sphingolipids were separated with two-dimensional TLC on glass plates (Merck, Germany) in the chloroform : methanol : water (65 : 25 : 4) solvent system—the first

direction; chloroform : acetone : methanol : acetic acid : water (50 : 20 : 10 : 10 : 5), in the second direction [16]. The lipids (100–200 μg) were applied on the plate. The chromatograms were developed by spraying with 5% sulfuric acid in ethanol with subsequent heating at 180°C. Individual markers and qualitative reactions with ninhydrin (for the presence of the amino group), Dragendorff reagent (for choline), and α-naphthol (for the carbohydrate groups) were used for lipid identification; the saponification method was used to determine the sphingolipid nature of glycolipids [15]. Neutral lipids were identified with individual markers: mono-, di-, and triacylglycerols, free fatty acids, sterols (ergosterol), and carbohydrates (Sigma, United States). Phosphatidylcholine (Sigma, United States) was used as the standard to determine the amount of phospholipids; a mixture of glycoceramides (Larodan, Sweden), for sphingolipids; ergosterol (Sigma, United States); for sterols. After densitometry, the quantitative analysis of the lipids was carried out using the Dens software (Lenkhrom, Russia) in the linear approximation mode with the calibration curves based on the standard solutions.

Analysis of the carbohydrate composition. Soluble mycelium sugars were extracted with boiling water four times for 20 min. The proteins were removed from the extract obtained [17]. The carbohydrate extract was further purified of charged compounds using a combined column with the Dowex-1 (the acetate form) and Dowex 50W (H^+) ion-exchange resins. The carbohydrate composition was determined with high-performance gas-liquid chromatography obtaining trimethylsilyl sugar derivatives [18]. α-Methyl-*D*-mannoside (Merck, Germany) was used as the internal standard. Chromatography was performed on a Kristall 5000.1 gas-liquid chromatograph (ZAO Khromatek, Russia) using a ZB-5 30 m × 0.32 mm × 0.25 μm capillary column (Phenomenex, United States) with a temperature program in the 130–270°C range at a rate of 5–6 degrees/min. Glucose, mannitol, arabitol, inositol, trehalose (Sigma, United States) were used as markers.

The experiments were made in three replicates; the results represent the typical experiment data. The scatter in the results did not exceed 10%; the main regularities coincided.

RESULTS

Influence of temperature on the growth of the fungus *R. miehei*. It was found as a result of studying the temperature characteristics of the fungus growth on a solid medium that the temperature limits of its growth were 28–55°C with a wide optimum in the 40–45°C range. Under the optimal temperature conditions, the submerged culture of the fungus grew in the form of 1–2 mm pellets with a wide loose margin consisting of unbranched hyphae.

No visible morphological changes were seen 1 h after exposure to high (51–53°C) temperatures (variant HS-1). After 3 h of exposure (variant HS-3), the pellets became denser with a narrow margin of loose mycelium, which gave evidence of the inhibition of growth processes.

It was established that for a lethal thermal shock, the trophophase submerged culture grown under the temperature optimum conditions was required to be heated for 20 min at 69°C with the resultant loss of viability by the fungus. The comparison between the HS-1 and HS-3 subjected to lethal HS and the control variants C-1 and C-3 did not reveal a significant difference between the variants. The findings indicate that the phenomenon of acquired heat resistance characteristic of mesophilic fungi is not inherent in the thermophilic fungus *R. miehei*.

The influence of HS on the composition of membrane lipids. The study of the composition of membrane lipids demonstrated that in the control variants, when *R. miehei* was grown by submerged culturing, the major lipids were represented by phospholipids (60–75% of the sum) and sterols (20–35%), whereas sphingolipids (3–5%) could be attributed to minor compounds (Fig. 1). PE, PC, and phosphatidic acids (PA) predominate in the phospholipid composition. Cardiolipins (CL), phosphatidylserines (PS), PI, lysophosphatidylethanol amines (LPE), and the unidentified phospholipids X_1–X_4 can be attributed to minor lipids.

When we compare variants C-1 and HS-1 (Fig. 1), it is seen that after 1-h HS, the PE, and PC shares in the membrane lipid composition decreased, and the PA and, especially, sterol (St) shares increased. The increase in HS duration up to 3 h (comparison between variants C-3 and HS-3) led to a further decrease in the relative PC content and an increase in the PA share. Note that no noticeable HS-induced changes occurred in the sphingolipid level.

The major membrane phospholipids were chromatographically isolated, and their fatty acid composition was investigated (table). It was found that the predominant fatty acids of the acylic chains of the membrane phospholipids PC, PE, and PA are palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1n9c}$), linoleic ($C_{18:2n6c}$), and α-linolenic ($C_{18:3n3}$). The acids $C_{12:0}$, $C_{14:0}$, $C_{14:1}$, $C_{15:0}$, $C_{16:1}$, $C_{17:0}$, and $C_{17:1}$ can be assigned to minor acids. In all PL, palmitic and oleic acids accounted for the greatest share of FA. Heat shock resulted in a drastic increase in the share of linoleic acid in the PE and PA composition and in the share of α-linolenic acid in the PC composition.

The action of HS on the composition of soluble cytosol carbohydrates. The study of the cytosol carbohydrate composition in the dynamics of growth of the fungus submerged culture of the control variants (Fig. 2) showed a high trehalose content (8–11% of dry mass) in the fungus cells beginning with the early trophophase (12 h) and up to the mid-idiophase (42 h). Apart from trehalose, glucose (2–3%) and a

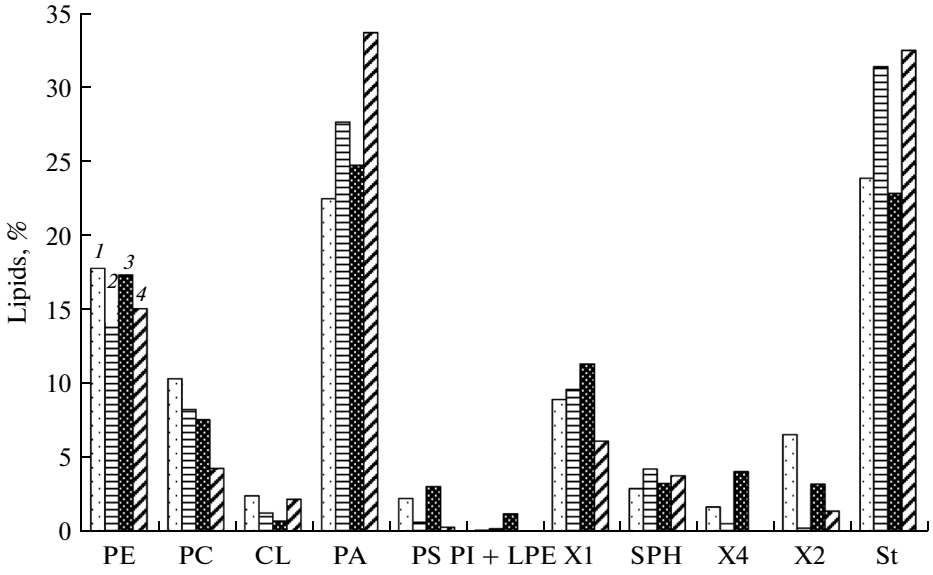

Fig. 1. Membrane lipid composition under heat shock conditions in *R. miehei* (% of the sum). Experimental variants: (*1*) C-1; (*2*) HS-1; (*3*) C-3; (*4*) HS-3.

trace amount of arabitol were identified. Heat shock of 1 h in duration did not increase the amount of trehalose (Fig. 3), and increased HS duration up to 3 h was accompanied by a substantial decline in the level of this disaccharide. No substantial variations in the amount of glucose were noted.

DISCUSSION

In order to clear up fungal responses to heat exposures, it is of interest to compare the results of changes in the lipid and carbohydrate composition obtained for the thermophilic fungus *R. miehei* with those of

Fatty acid composition of the major phospholipids under heat shock conditions at the trophophase stage (% of the sum) of *R. miehei*

Fatty acids	C-3			HS-3		
	PE	PC	PA	PE	PC	PA
$C_{12:0}$	1.3	1.1	–	–	–	–
$C_{14:0}$	2.0	1.7	–	–	–	–
$C_{14:1}$	1.6	0.8	–	4.6	6.9	–
$C_{15:0}$	3.7	0.8	–	–	–	3.8
$C_{16:0}$	30.5	31.9	28.1	20.5	32.4	32.8
$C_{16:1}$	–	0.9	–	–	–	–
$C_{17:0}$	–	1.1	–	–	–	–
$C_{17:1}$	–	1.1	–	–	–	–
$C_{18:0}$	9.3	16.9	27.2	10.3	17.6	25.1
$C_{18:1n9c}$	25.9	25.8	27.6	18.0	17.4	15.9
$C_{18:2n6c}$	16.5	9.1	5.8	39.5	5.5	15.7
$C_{18:3n3}$	9.2	8.8	11.3	6.9	20.2	6.7
Unsaturation degree	0.88	0.73	0.73	1.22	0.96	0.68

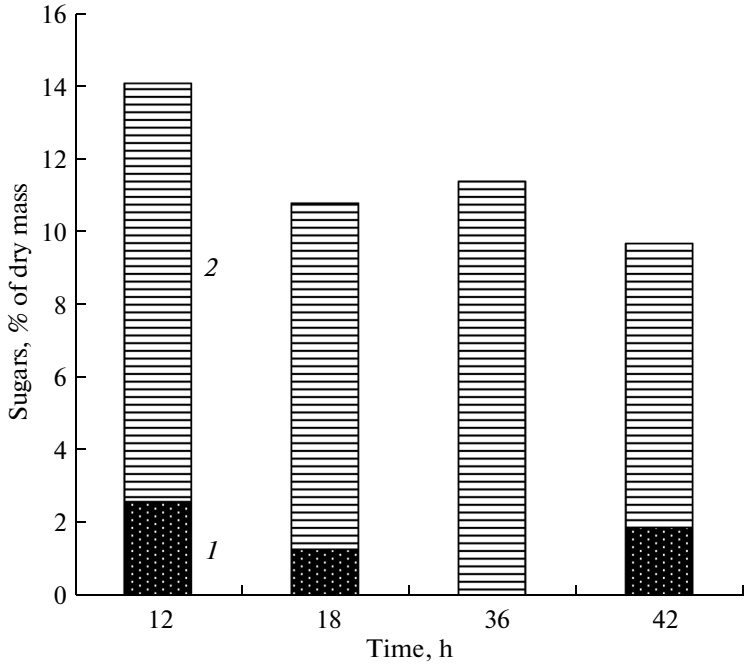

Fig. 2. Composition of the major soluble cytosol carbohydrates in the trophophase of *R. miehei* under heat shock conditions. (*1*) glucose, (*2*) trehalose.

mesophils. Thus, the study of stress response to HS in three mesophilic fungi of different taxonomic position (*Aspergillus niger, Pleurotus ostreatus*, and *Cunninghamella japonica*) and the study of the membrane lipid composition showed that there existed universal changes—the increase in the trehalose level and in the share of phosphatidic acids (against the background of the PC and PE share decrease)—as well as individual ones—the increase in the relative number of sterols and sphingolipids [19]. The thermophilic fungus *R. miehei* also revealed similar changes in the membrane lipid composition in response to HS: the shares of PA and St increased and those of PE and, especially, PC decreased. Additionally, no decrease in the unsaturation degree of the fatty acids of the major membrane phospholipids exposed to HS was observed, similar to mesophils. Thus, the HS-induced peculiarities of the membrane lipid composition characteristic of mesophilic fungi are also inherent in thermophils. Quite different regularities are observed in the cytosol carbohydrate composition. As distinct from the mesophils containing trace amounts of trehalose under the growth optimum conditions, the cells of the thermophilic fungus revealed a high trehalose level (8–11% of dry mass) under the same optimal conditions, beginning with the early growth stages, which agrees with the literature data on the high trehalose level (3–3.5%) in another thermophilic fungus *M. thermophila* [10]. Our investigations showed that 1-h HS exposure did not cause the amount of trehalose to increase in *R. miehei*, but after 3 h of exposure it decreased twofold. For comparison, in the mesophilic fungus *A. niger*, a high trehalose level was observed only under HS conditions and maintained at a level of 6% for 6 h of exposure [20]. Thus, the changes in the cytosol carbohydrate composition influenced by HS are fundamentally different in mesophils and thermophils. Interestingly, *A. niger* exposed to HS revealed a noticeable increase in the level of sphingolipids (SPH) exceeding that of PA. Note that the sterol level did not change on exposure to even 9-h thermal stress, whereas for the thermophilic fungus *R. miehei* the amount of St noticeably increased under HS condi-

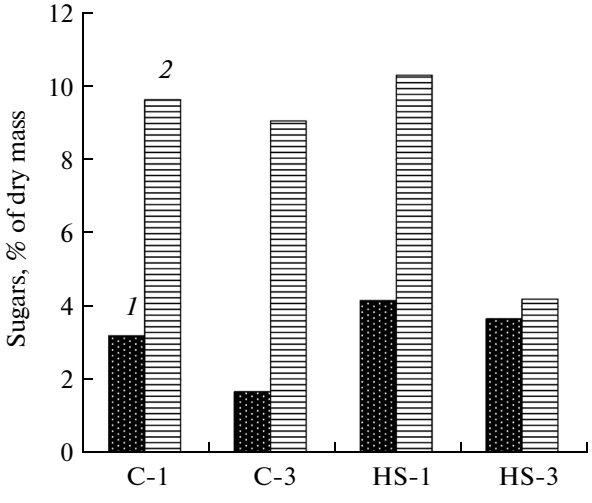

Fig. 3. Major cytosol carbohydrates in the cells of *R. miehei* under heat shock conditions; (*1*) glucose, (*2*) trehalose.

tions and the SPH content remained at a low level. Sphingolipids, together with sterols, are suggested to form lipid rafts in the plasma membrane of eukaryotic cells (mammals, yeasts, and mycelial fungi) where they are involved in delivery of specific proteins. In addition, the fungi have more complex structures rich in sterols [21, 22]. They are larger than the lipid rafts, situated in the external membrane layer, and take part in protein grading.

The main distinctive feature of the thermophilic fungus in question, compared to mesophilic fungi, is a high trehalose content under the temperature optimum conditions favorable for growth. Moreover, the amount of trehalose does not increase in response to HS, as in mesophilic fungi, but decreases. Another distinction is the absence in *R. miehei* of the phenomenon of acquired heat resistance inherent in mesophilic fungi.

Analyzing the changes in the membrane lipid component under HS conditions, we may conclude that, in contrast to the homeoviscous adaptation hypothesis [1], no decrease in the unsaturation degree of the acyl chains of the major membrane phospholipids was observed on heat shock exposure. On the contrary, in a number of cases (for PC and PE) it increased. An increase in the PE/PC ratio in *R. miehei* and a rise in the PA content in HS result in the increase in the share of nonbilayer lipids, which runs counter to Hazel's homeophasic adaptation hypothesis [2], according to which adaptation to heat shock occurs due to the maintenance of a certain ratio between the stabilizing cylinder-shaped (PC) lipids forming the bilayer and the destabilizing cone-shaped (PE, PA) lipids. The third hypothesis links membrane defense under HS conditions to thermoprotective compounds, trehalose, sterols, glycolipids, etc. [3]. In the fungus studied, we revealed a rise in the relative sterol content, but no increase in the trehalose level was noted, which can be explained by the high cell content of this disaccharide (up to 11% of dry mass) when growth was under optimum temperature conditions. The fact that the increase in HS duration results in the trehalose level decrease might evidence the inhibition of trehalososynthase. Interestingly, the fungus does not reveal the appearance of acquired heat resistance due to the effect of HS, which may be linked to the impossibility of an additional increase in trehalose synthesis. In the final analysis, we may conclude that the mechanisms of adaptation to HS significantly differ in thermophilic and mesophilic fungi.

The question of the role of PA in thermophils has long been discussed in the literature. The special role of this compound was suggested based on the results of the study of the thermophilic fungus *Humicola grisea* var. *thermoidea* whose share of PA accounted for 35% of the sum of phospholipids [7]. However, further investigations did not confirm such a feature for thermophilic fungi. For example, in four thermophilic fungi—*Thermomyces lanuginosus* (syn. *Humicola lanuginosa*), *Thermoascus aurantiacus, Malbranchea pulchella* var. *sulfurea*, and *Lichteimia ramosa* (syn. *Absidia ramosa*)—the share of PA constituted up to 6% [23], while in *M. thermophila*, an insignificant amount of PA was noted [8]. However, three mesophilic fungi showed an increase in the level of PA against the background of the share of the major membrane phospholipids (PC and PE) being decreased on long-term exposure to HS [19]. Further studies with marked sodium acetate showed that, in *A. niger*, under conditions of HS the mark was washed out of PC and PE after removal of the marked substrate and accumulated in PA, which indicates the origin of PA as a result of hydrolysis of PC and PE by phospholipase D [24]. The results of this study showed that in *R. miehei*, the share of PA was high enough in the process of growth but, in a way similar to mesophils, it additionally increased on exposure to HS. The most probable function of PA under conditions of HS seems to be their involvement in the processes of endo- and exocytosis, which is determined by the ability of PA to form domains and to take part in the formation of membrane curvatures [25, 26].

The results of this study allowed us to conclude that the mechanisms of defense against HS in mesophilic and thermophilic fungi differ in part. Against the background of similarity to changes in the membrane lipid composition under HS conditions, substantial changes are observed in the carbohydrate composition, namely, in thermophils with their high trehalose level under the temperature optimum; its content is seen to decrease on HS exposure. The absence of the phenomenon of acquired heat resistance is a peculiarity that distinguishes thermophils from mesophils, which, according to the study data, may be attributed to reduction in the trehalose level.

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