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EXPERIMENTAL ARTICLES

Respiratory Activity of Yeast *Yarrowia lipolytica* under Oxidative Stress and Heat Shock

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Abstract—Heat shock (45°C) and the effect of oxidants (H_2O_2) resulted in a decrease of the respiratory activity of yeast cells and their survival rate. Increased resistance to stress effects after mild heat treatment (37°C) or treatment with a nonlethal dose of oxidants (0.5 mM H_2O_2) for 60 min) was accompanied by appearance of an alternative (cyanide-resistant) oxidative pathway in the mitochondria, which promotes survival due to retention of the capacity for ATP synthesis in the first coupling point at the level of endogenous NADH dehydrogenase. The alternative oxidative pathway is more resistant to the effect of stressors that disrupt electron transfer in the cytochrome site of the respiratory chain.

Key words: yeast *Yarrowia lipolytica*, heat shock, oxidative stress, survival, respiratory activity, inhibitor analysis, alternative oxidase

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Various stress effects are known to lead to development of adaptive mechanisms in microorganisms, which strengthens their resistance to unfavorable environmental factors [1]. The influence of stressors on cells is directly or indirectly caused by formation of reactive oxygen species (ROS), whose effect results in cell death [2]. Analysis of the literature [3–7] indicates that mitochondria and, in particular, the respiratory chain are one of the targets of ROS effect. ROS induce peroxide oxidation of cardiolipin, the phospholipid indispensable for the functioning of cytochrome oxidase. Furthermore, ROS can directly inactivate electron transfer via the respiratory chain, ATPase, transhydrogenase, and other proteins [4, 5, 7].

Literature data also demonstrate that the action of stressors leads to the stimulation of cellular genetic activity, and, as a result, the emergence of shock proteins, participating in protection from the lethal effects of ROS and high temperature [3, 8]. Mitochondrial localization of stress-induced proteins indicates the participation of mitochondria in cell response to stressors [3].

Many eukaryotic microorganisms with aerobic metabolism (not fermenting glucose), under certain stress conditions are able to use a pathway of electron transfer alternative to the main cytochrome respiratory chain, which is also called alternative oxidase [8]. The latter is localized in mitochondria, branched off from the main respiratory chain at the level of ubiquinone, and is specifically inhibited by derivatives of benzohydroxamic acid (BHA). Oxidation of the substrates that supply reduced equivalents directly at the level of ubiquinone via alternative oxidase is uncoupled with energy storage in a form available to the cell. Oxidation of the substrates via endogenous NADH dehydrogenase localized in the inner side of the interior mitochondrial membrane is coupled with ATP formation in the first point of coupling.

It has been demonstrated [6] that in *Neurospora crassa* heat shock (45°C) resulted in a decrease of respiratory activity and deenergization of the mitochondrial membrane. After 40-min incubation at the same temperature (+45°C), cell respiration recovers, but becomes resistant to KCN. After the action of nonlethal temperature (30°C) on fungal cells, apart from many classical cytosol heat shock proteins, a protein (38 kDa) characteristic of alternative cyanide-resistant oxidase was discovered [9].

The goals of the present work were to study the respiratory activity of *Y. lipolytica* at heat shock and oxidative stress, to determine the participation of alternative cyanide-resistant oxidase in adaptive cell response to stress effects, and to assess the influence of this enzyme on yeast survival.

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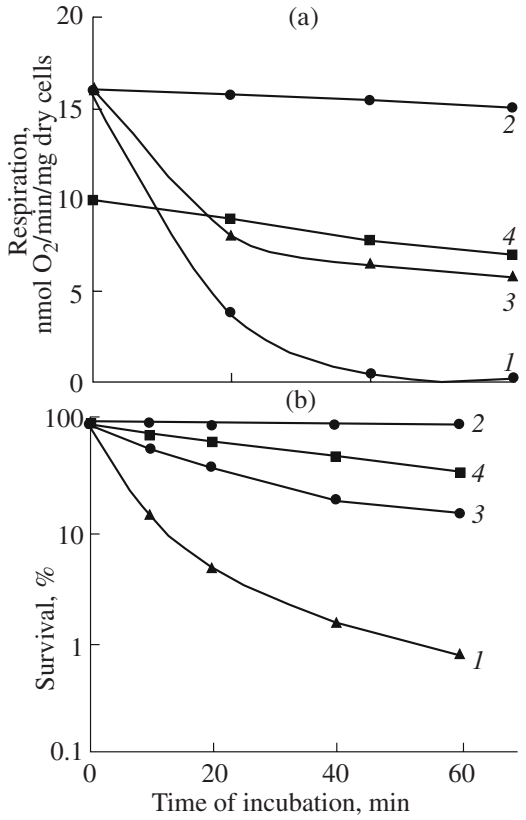

Fig. 1. Respiratory activity (a) and survival (b) of *Y. lipolytica* cells under oxidative stress. *1, 2, 3*, cells of the exponential growth phase; *4*, cells of the stationary growth phase; *1, 4*, 120 mM H_2O_2; *2*, 0.5 mM H_2O_2; *3*, 120 mM (pretreatment with 0.5 mM H_2O_2 for 60 min).

MATERIALS AND METHODS

The strain *Yarrowia lipolytica* VKM Y-2378 obtained from the All-Russian Collection of Microorganisms of the Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, was used in this work. The strain is able to develop an alternative cyanide-resistant oxidative pathway under certain conditions [10]. The cultivation was performed at 29°C in 750 ml flasks containing 100 ml of Reader medium [11] with glucose (1%) on a shaker at 200 rpm. Yeast biomass was evaluated by optical density at 540 nm. The cells were washed with sterile distilled water and re-suspended in 50 mM *Tris*-phosphate buffer (pH 7.0).

Oxidative stress conditions were modeled by incubation of cell suspensions in the exponential (10–12 h) and stationary (24 h) growth phases in the presence of H_2O_2 (120 mM). For adaptation, cells were treated with nonlethal doses of H_2O_2 (0.5 mM) for 60 min [11].

Heat shock conditions were modeled by incubation of cells of different growth phases at 45°C. Mild heat pretreatment (adaptation to heat shock) was achieved by cell incubation at 37°C for 60 min.

Oxygen consumption was measured on a polarograph using a Clark-type platinum electrode covered with Teflon film. During the measurements, the temperature was maintained at 20–22°C.

As inhibitors of respiration, antimycin A (5 μM), BHA (5 mM), and KCN (1 mM) were employed.

To measure yeast respiration in the presence of 120 mM H_2O_2, 5 μg of catalase was added to 10 ml of cell suspension and quickly (during 1 min) precipitated at 10000 *g*. The precipitate was resuspended in 10ml buffer and used for respiration measurements.

Cell survival was determined by inoculation of petri dishes with wort agar. The colonies were counted after 48–72 h cultivation at 29°C. The data represent the results of three independent experiments.

In the work commercial preparations (Sigma) of H_2O_2, antimycin A and BHA were used.

RESULTS AND DISCUSSION

The data on the changes of cell respiratory activity and survival at oxidative stress are given in a comparative aspect in Fig. 1a and 1b. As seen in Fig. 1a, (curve *1*), in the presence of H_2O_2 at the concentration 120 mM (earlier established in [11] as lethal) the respiratory activity of the exponential phase cells drastically dropped: after 20 min, by 70%, and after 40 min, almost to zero. Cell incubation at low doses of the oxidant (0.5 mM H_2O_2) led to neither decrease of the respiration rate nor decrease in yeast survival (Figs. 1a and 1b, curves *2*).

Yeast cell pretreatment by nonlethal doses of oxidants [11, 12] is known to result in an increase of cell survival under oxidative stress (120 mM H_2O_2).

In this work it was also shown that pretreatment of exponential phase cells with a nonlethal dose of the oxidant (0.5 mM H_2O_2 for 60 min) under oxidative stress conditions results not only in cell survival but in a higher level of respiration as well (Figs. 1a and 1b, curves *3*) than in non-adapted cells (Fig. 1a and 1b, curves *1*).

Respiration of cells from the stationary growth phase was less sensitive to the lethal effect of H_2O_2 (Fig. 1b, curve *4*) and was comparable with such in adapted cells from the exponential growth phase (Fig. 1b, curve *3*).

The activity of cell respiration and survival correlated in all the above cases in the different growth phases (Fig. 1a and 1b).

Heat shock (45°C) also led to a sharp drop in survival and the respiratory activity of cells from the exponential growth phase (Fig. 2). Thus, the rate of oxygen consumption under heat shock was less than 10% of the initial level after 5 min and was completely absent after 15 min (Fig. 2a, curve *1*). The cell survival dropped almost to zero (Fig. 2b, curve *1*). Yeast incubation at 37°C for 30 min (mild heat treatment) had almost no

effect on cell respiration and survival (Fig. 2a and 2b, curves *2*), but appreciably increased their resistance to subsequent treatment at higher temperature (curves *3*). Similar to the oxidative stress, cells from the stationary growth phase (curves *4*) exhibited higher resistance to heat treatment (in terms of survival and respiratory activity) than those from the exponential growth phase (curves *1*).

The results given in Figs. 1 and 2 indicate that oxidative stress and heat shock drastically decreased the respiratory activity. This allows us to suggest that the respiratory chain was one of the targets of the damaging factors under heat shock and oxidative stress.

As was mentioned in the introduction, in many yeasts, including *Y. lipolytica*, an alternative cyanide-resistant oxidative pathway emerges under stress conditions [10]. To define alternative oxidase activity of *Y. lipolytica* depending on stress effect and yeast physiological state, an inhibitory respiration assay was performed. As can be seen from the data of the table, the respiration of exponential phase yeast was sensitive to cyanide and antimycin A and in their presence was inhibited by almost 100%. On the contrary, respiration of the stationary growth phase cells was not inhibited by cyanide, but appreciably accelerated in its presence. The mechanism of respiration activation by cyanide has been described previously [10]. Joint application of KCN and BHA resulted in complete inhibition of respiration. These results indicate that along the cytochrome chain, an alternative cyanide-resistant oxidative pathway functions in cells of the stationary growth phase. It also follows from the table that incubation of the cells from the exponential growth phase in the presence of a nonlethal dose of H_2O_2 (0.5 mM) or mild heat pretreatment (at 37°C for 60 min) led to a decrease in the inhibitory effect of KCN on respiration. Respiration was completely suppressed at joint application of KCN and BHA, which also indicates the emergence of an alternative oxidative pathway.

Figs. 3 and 4 represent the data on yeast survival depending on the functional activity of the main cytochrome respiratory chain or the alternative cyanide-resistant pathway under stress conditions. For this purpose, cells in which the both oxidative pathways were active (table) were subject to the effect of H_2O_2 (120 mM) or temperature (45°C) in the presence of antimycin A or BHA. In the presence of antimycin A, which inhibits the main respiratory chain at the level of cytochrome *b*, only alternative oxidase was active; in the presence of BHA, which inhibits the alternative pathway, only the cytochrome chain was functioning. In all tested stress conditions, cell survival was lower in the presence of inhibitors (curves *2* and *3*) than in the

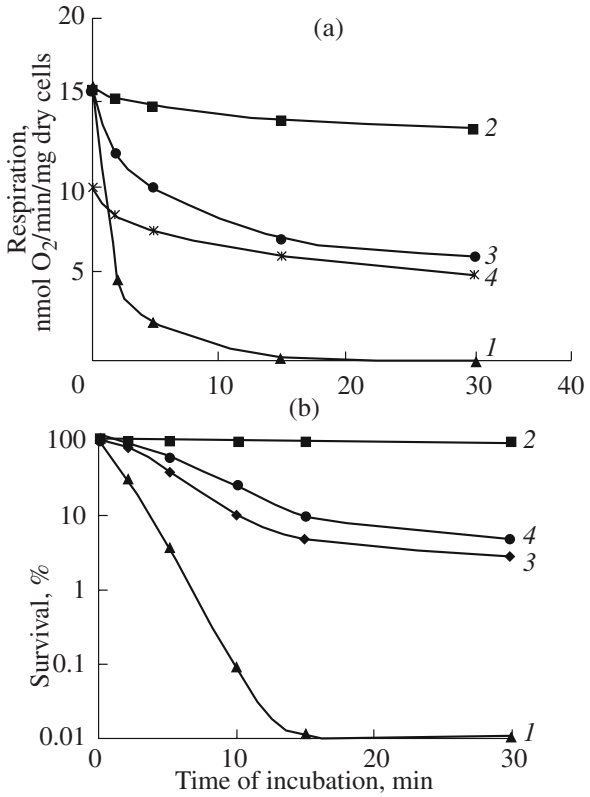

Fig. 2. Respiratory activity (a) and survival (b) of *Y. lipolytica* cells under heat stress. *1, 2, 3*, cells of the exponential growth phase, *4*, cells of the stationary growth phase; *1, 4*, 45°C; *2*, 37°C; *3*, 45°C (preincubation at 37°C for 60 min).

Respiratory activity in the cells of *Y. lipolytica*

Experimental conditions	Growth phase	Respiration, nmol O_2/(min mg dry cells)	Inhibition of respiration, %			Activity of alternative oxidase, nmol O_2/(min mg dry cells)
			KCN, 1 mM	Antimycin A, 5 μM	KCN (1 mM) + BHA (5 mM)	
Without treatment	Exponential	15.8	98	98	100	0
Without treatment	Stationary	10.9	+190*	+160*	96	32
Heat pretreatment (37°C, 60 min)	Exponential	16.8	70	68	98	5.5
Treatment with H_2O_2 (0.5 mM, 60 min)	Exponential	16.5	60	70	97	6.5

* The symbol "+" indicates a stimulating effect of the inhibitor.

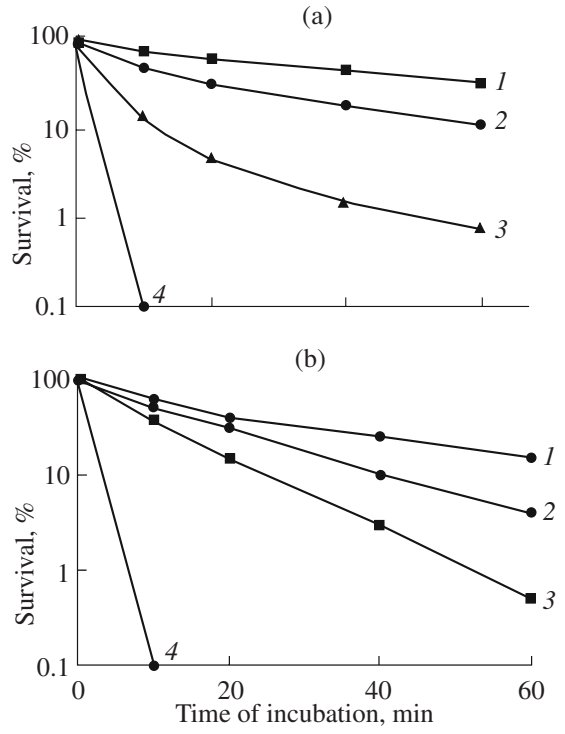

Fig. 3. Effect of respiration inhibitors on cell survival of *Y. lipolytica* in the stationary (a) and exponential (b) growth phases under oxidative stress (120 mM H_2O_2). Cells of the exponential growth phase were adapted by preincubation in the presence of 0.5 mM H_2O_2 for 60 min. *1*, control (without inhibitors); *2*, antimycin A (5 μM); *3*, BHA (5 mM); *4*, antimycin A + BHA.

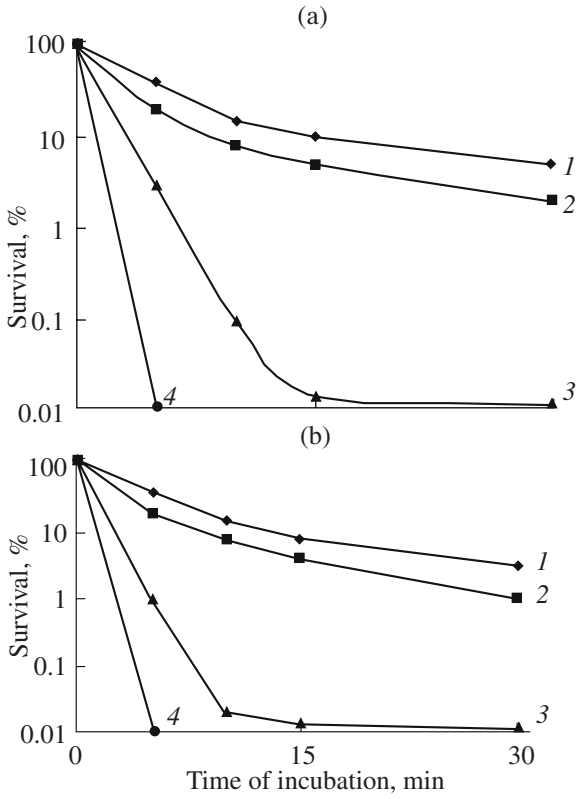

Fig. 4. Effect of respiration inhibitors on survival of cells of *Y. lipolytica* of the stationary (a) and exponential (b) growth phases under heat stress. Cells of the exponential growth phase were adapted by preincubation at 37°C for 60 min. *1*, control (without inhibitors); *2*, antimycin A (5 μM); *3*, BHA (5 mM); *4*, antimycin A + BHA.

control (without inhibitors, curves *1*). It is remarkable that the functioning of the alternative oxidase provided higher cell survival (curves *2*) than the functioning of the main respiratory chain (curves *3*). This pattern was found in cells of both growth phases, the stationary and the exponential (adapted cells). However, in the latter case the parameters of survival were somewhat lower than of those in cells of the stationary growth phase. This phenomenon possibly results from the fact that the activity of the alternative oxidase in stationary cells was five times higher than in adapted cells from the exponential growth phase (Table). Understandably, the cells were practically unviable in the presence of both inhibitors (curves *4*).

The data presented in the table and Figs. 3 and 4 indicate that the respiratory chain of *Y. lipolytica* plays an important role in the preservation of cell viability at oxidative stress and heat shock.

Literature data on the effect of inhibitors of respiration on cell survival noticeably differ depending on the yeasts tested. It was shown that at heat shock (50°C), the presence of cyanide or sodium azide appreciably increased destruction of *Rhodotorula rubra* and *Debaryomyces vanrij* cells but had no negative effect on *Saccharomyces cerevisiae*, and the addition of azide even increases their heat resistance (an increase in survival was observed) [13]. The authors explain these facts in this way: yeasts *R. rubra* and *D. vanrij* are characterized by aerobic metabolism (do not ferment glucose) and store energy as ATP only when the phosphorylation respiratory chain is functioning, while *S. cerevisiae* are able to obtain energy via glucose fermentation [13].

Earlier, we have demonstrated that the activity of antioxidant enzymes in *Y. lipolytica* (catalase, superoxide dismutase, glucose-6-phosphate dehydrogenase, and glutathione reductase) increased in the process of adaptation to stress conditions [11].

The presented data unequivocally demonstrate that, apart from increasing the activity of antioxidant enzymes, the yeast are also able to induce an alternative (cyanide-resistant) oxidase, which provides for survival by retention of the ability for ATP synthesis at the first point of coupling at the level of endogenous NADH dehydrogenase. It is noteworthy that the alternative oxidative pathway is more resistant to the effect of stres-

sors disrupting electron transfer in the cytochrome site of the respiratory chain.

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