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EXPERIMENTAL ARTICLES

Adaptation of the Yeast *Yarrowia lipolytica* to Ethanol

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Abstract—Resistance of the yeast *Yarrowia lipolytica* to ethanol stress was studied under different ethanol concentrations and treatment duration. Cell viability was shown to increase in the variants including preliminary treatment with small doses of ethanol, oxidants, or soft thermal exposure. The study of the respiratory activity under ethanol stress revealed the involvement of an alternative cyanide-resistant oxidase in the adaptive response of the cells. The level of intracellular cAMP decreased in response to the action of ethanol, which correlated with increased activity of the antioxidant systems (catalase, superoxide dismutase, glucose-6-phosphate dehydrogenase, glutathione reductase) and NAD^+-dependent alcohol dehydrogenase.

Key words: Yarrowia lipolytica, stress, adaptation, ethanol, respiratory activity, inhibitor analysis, alternative oxidase, cAMP, NAD^+-dependent alcohol dehydrogenase.

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Ethanol in high concentrations is able to suppress the physiological activity of yeast cells [1–4].

Ethanol toxicity is associated with its ability to suppress the biosynthesis of macromolecules, denature the cytoplasmic proteins, reduce the activity of glycolytic enzymes [2, 5–7], disturb the processes of the transport of ions and metabolites across the plasma membrane [5–7], and to change the membrane's lipid composition [4, 8–10]. The primary effect of ethanol results from the increased anion and proton permeability of the cytoplasmic membrane accompanied by its deenergization (drop of ΔpH and E_m) [5–7, 11] and decrease of intracellular pH [11].

The exposure of *Saccharomyces cerevisiae* to suboptimal temperature or ethanol was previously shown to result in the appearance of mutants with impaired respiratory activity [1, 2]. The effect of these stressors may be associated with a disturbance of the mitochondrial electron transfer chain resulting in enhanced formation of reactive oxygen species (ROS) resulting in oxidative damage of proteins, lipids, and DNA. The issues concerning the effect of stressors on the respiratory activity of yeasts are poorly understood.

The goal of this work was to reveal the changes in the mitochondrial respiratory chain, to determine the activity of antioxidant enzymes, and to study the dynamics of cAMP during adaptation of the yeast *Yarrowia lipolytica* to different ethanol concentrations.

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MATERIALS AND METHODS

The yeast *Yarrowia lipolytica* VKM Y-2378 was obtained from the All-Russian Collection of Microorganisms, Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences. The yeast was cultivated at 29°C in 750-ml flasks containing 100 ml of the Rider medium [12] with glucose (1%) on a shaker (200 rpm). The yeast biomass was assessed by the optical density of the culture at a 540 nm (Specol 21).

Stress was induced with different ethanol concentrations applied to cell suspensions from the exponential (10–12 h) and stationary (24 h) growth phases. For this purpose, the cells were washed from the medium with sterile distilled water, resuspended in 50 mM Tris–phosphate buffer (pH 7.0), and incubated in the presence of different ethanol concentrations.

The cells were adapted by pretreatment with small doses of ethanol (1%, 60 min) or oxidants (0.5 mM H_2O_2, 60 min), or by incubation at 37°C for 60 min.

The survival rate was determined by plating on wort agar. Colonies (CFU) were counted after 48–72 h of cultivation at 29°C.

The oxygen consumption rate was measured with a Clark-type platinum electrode closed by a Teflon film. The final volume of a sample was 2 ml; measurement temperature was 20–22°C. The concentration of oxygen dissolved in the medium was assumed equal to 250 μM. Respiration activity was expressed in nmol O_2 min^{-1} mg^{-1} of dry cells.

The cAMP was extracted from the cells with perchloric acid (5%). The extract was neutralized with 5 N KOH under intensive stirring. The cell precipitate was

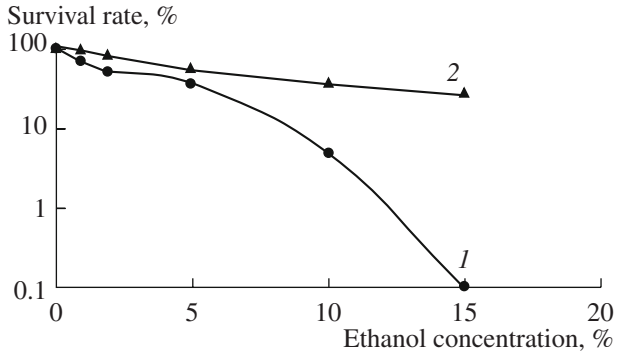

Fig. 1. Resistance of *Y. lipolytica* to different ethanol concentrations: cells from the exponential growth phase (*1*); cells from the stationary growth phase (*2*).

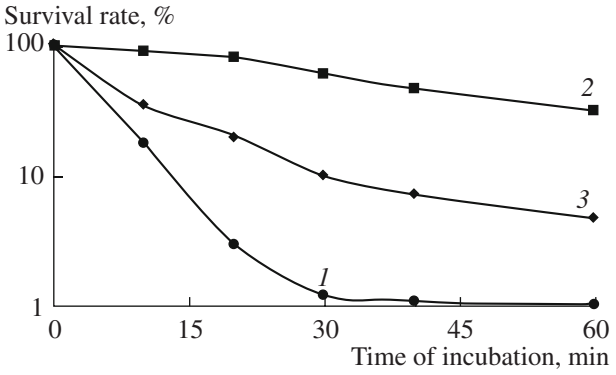

Fig. 2. Resistance of *Y. lipolytica* from the exponential growth phase to ethanol (15%) depending on pretreatment of cells with small doses of alcohol: without pretreatment (control) (*1*); pretreatment with ethanol (1%, 60 min) (*2*); pretreatment with ethanol (5%, 60 min) (*3*).

removed by centrifugation at 15000 *g* for 60 min. The extract was stored at –15°C. cAMP content was determined by the standard procedure with an Amersham kit.

Cell extracts for the determination of enzyme activities were obtained as follows. The cells were washed twice with distilled water and suspended in 50 mM Tris–phosphate buffer (pH 7.0) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, followed by cell disintegration in a French press. The homogenate was centrifuged at 105000 *g* for 60 min. The precipitate was discarded and the supernatant was used for determination of enzyme activities.

The activity of antioxidant enzymes (superoxide dismutase (SOD), catalase, glucose-6-phosphate dehydrogenase, and glutathione reductase) was determined as described previously [12]. The activity of NAD^+-dependent alcohol dehydrogenase was measured by NAD^+ reduction, $E_{340} = 6.22\ mM^{-1}\ cm^{-1}$ [13].

Protein content was assayed using the biuret reagent.

Spectral studies were performed in a Shimadzu spectrophotometer (Japan).

The commercial preparations of H_2O_2, antimycin A, and benzhydroxamic acid (BHA) (Sigma) were used in the work.

RESULTS AND DISCUSSION

Fig. 1 presents the data on the survival rate of *Y. lipolytica* cells grown on glucose (1%) after exposure to various ethanol concentrations for 60 min. It can be seen that increased alcohol concentrations resulted in a noticeable decrease in the viability of the cells from the exponential growth phase (curve *1*). The lethal ethanol concentration was 15%. The cells from the stationary growth phase were more resistant to ethanol (curve *2*).

Fig. 2 shows that pretreatment of the exponential-phase *Y. lipolytica* cells with small doses of ethanol results in their resistance to higher alcohol concentration (15%). Incubation of the cells in the buffer without ethanol did not increase the survival rate in the presence of the lethal dose of ethanol.

Ethanol concentrations for adaptation (1 and 5%) were selected in accordance with the data presented in Fig. 1. The greatest positive effect was obtained after pretreatment of *Y. lipolytica* with 1% ethanol (Fig. 2, curve *2*).

An increase of ethanol tolerance resulting from preliminary treatment of the cells with a small dose (4–6%) has been demonstrated for *S. cerevisiae* [1]. Higher ethanol concentrations required for a positive adaptation effect probably result from the ability of this yeast to ferment glucose to ethanol.

Pretreatment of *Y. lipolytica* cells with ethanol (1%, 60 min) also proved to increase their resistance to oxidants and temperature. As one can see from Figure 3, the cells pretreated with ethanol (1%, 60 min) were more viable under heat (45°C) and oxidative (120 mM H_2O_2) stresses (curves *3* and *4*, respectively) than non-adapted cells (curves *1* and *2*).

Pretreatment of *Y. lipolytica* with a nonlethal dose of H_2O_2 (0.5 mM, 60 min) resulted in increased cell resistance to the high dose of ethanol (15%). In this case, about 15% of the cells remained viable (Fig. 4, curve *2*). The maintenance of cell viability at a higher level was observed after soft thermal pretreatment (37°C, 60 min). In this case, about 35% of the cells remained viable (Fig. 4, curve *3*).

Thus, *Y. lipolytica* showed a marked capacity for cross adaptation when one type of stress resulted in development of resistance to other stress impacts. These results are in agreement with the data obtained for *S. cerevisiae* [14, 15]. Probably, yeasts have a common stress center (STRE), activation of which results in the development of adaptive processes. At the same time, a group of genes is transcribed, the main function

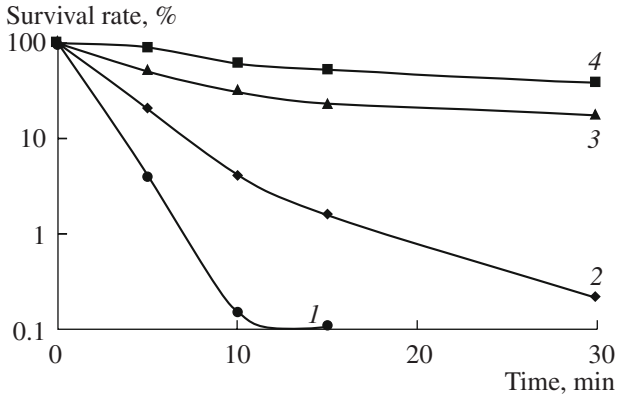

Fig. 3. The effect of pretreatment with ethanol (1%) on the survival of *Y. lipolytica* from the exponential growth phase under heat (*1, 3*) and oxidative (*2, 4*) stresses: without pretreatment (control) (*1, 2*,); pretreatment with ethanol (1%, 60 min) (*3, 4*).

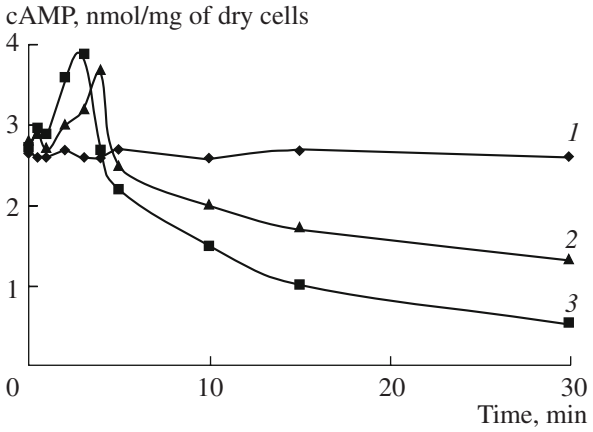

Fig. 5. The content of cAMP in *Y. lipolytica* cells under ethanol stress: without ethanol (control) (*1*); 1% ethanol (*2*); 15% ethanol (*3*).

of which is integrated protection of the cells from several different stressors.

In *S. cerevisiae*, transcription of some genes within the STRE group is known to be under the negative control of protein kinase A [14, 16, 17]. A strong correlation was demonstrated between the activity of the cAMP-dependent protein kinase and heat tolerance. Mutant strains with the low activity of protein kinase A were considerably more heat-resistant [18], whereas the mutants with the high constitutive activity of protein kinase A were heat-sensitive under all stress conditions [17, 18].

It can be surmised that cAMP is one of the triggering signals for development of adaptive mechanisms in response to stress impacts. Previously [19] it has been shown that the level of this nucleotide in *Y. lipolytica* decreased under oxidative and thermal stresses.

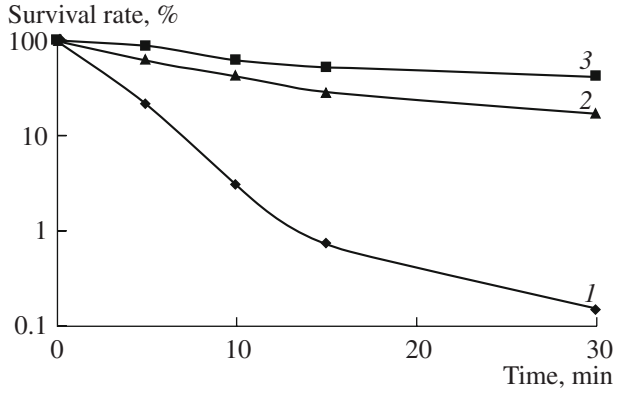

Fig. 4. Cross resistance of *Y. lipolytica* from the exponential growth phase under ethanol stress (15%): without pretreatment (control) (*1*); pretreatment with a nonlethal dose of H_2O_2 (0.5 mM, 60 min) (*2*); thermal pretreatment (37°C, 60 min) (*3*).

Fig. 5 shows the data on the change of the cAMP content in *Y. lipolytica* in the presence of ethanol. The initial content of cAMP is within 2.6–2.8 nmol per 1 mg of dry cells. Under the action of ethanol in the concentrations of 1% (Fig. 5, curve *2*) and 15% (Fig. 5, curve *3*), a short-term increase of the cAMP level occurred (with the maximum at 3 min) followed by a drop below the initial level.

As has already been mentioned in the Introduction [5–7], addition of ethanol into the yeast incubation medium results in a decrease of the electrochemical potential of the cytoplasmic membrane and therefore in impaired transport of various metabolites and decrease of intracellular pH.

For completeness, we have studied the toxic effect of ethanol on the respiratory activity of *Y. lipolytica*.

The study of the ethanol effect on respiration (Fig. 6) showed that low ethanol concentration (1%) had practically no effect on the rate of oxygen consumption by the cells from the exponential growth phase (curve *2*). In the presence of 5% ethanol, the respiratory activity of *Y. lipolytica* remained at a level of 70–75% (curve *3*), while an increase of alcohol concentration to 15% resulted in cell respiration decreasing practically to zero after 20 min of incubation (curve *4*).

The inhibitor analysis of respiratory activity was performed to reveal an alternative oxidase in *Y. lipolytica* under ethanol stress. The results are presented in Table 1. It can be seen that respiration of *Y. lipolytica* cells from the exponential growth phase is highly sensitive to both cyanide and antimycin A; in their presence, it is almost completely inhibited. The rate of oxygen consumption by the cells from the stationary phase was not inhibited by cyanide and was even noticeably accelerated in its presence; the joint action of these inhibitors completely suppressed respiration, which

was evidence of the existence of a cyanide-resistant oxidase.

Adaptation of the cells to ethanol (1%, 60 min) was followed by a decrease of the inhibiting effect of KCN on cell respiration; the latter was also completely suppressed by a joint action of KCN and BHA, supporting the emergence of an alternative oxidation pathway.

Thus, the results of the inhibitor analysis demonstrate that the resistance of respiration of adapted cells from the exponential and stationary growth phases to the lethal ethanol concentration (15%) is determined by the presence of an alternative cyanide-resistant oxidation pathway. This pathway seems to be more resistant to the action of high ethanol concentrations than the main cytochrome respiratory chain. The alternative oxidase is branched from the main respiratory chain at the level of coenzyme Q (ubiquinone) and carries the reducing equivalents from complex I (exogenous NADH dehydrogenase) and complex II (exogenous NADH dehydrogenase, succinate dehydrogenase, a-glycerophosphate dehydrogenase) to oxygen [20]. Consequently, one may suppose that the cytochrome region from ubiquinone to cytochrome oxidase is the weak link in the main respiratory chain in response to the effect of ethanol. Cytochrome *c*, which is a relatively mobile component of the respiratory chain, may be the most probable candidate for the weak link. Probably, as a result of the disturbance of membrane permeability observed under the effect of ethanol, the low-molecular cytochrome *c* diffuses through the mitochondrial outer membrane into the cytosol and the respiratory chain stops functioning.

The decrease of the activity of cell respiration in the presence of ethanol (Fig. 6) associated with impaired electron transfer in the cytochrome region of the respiratory chain suggests increased ROS production [1, 2], which essentially contributes to the death of cells.

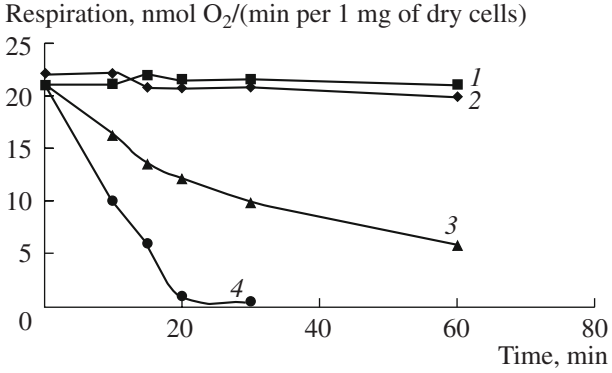

Fig. 6. The effect of ethanol on respiration of *Y. lipolytica* from the exponential growth phase: control (*1*); 1% (*2*); 5% (*3*); 15% (*4*).

Table 2 shows the data of measurement of the activities of the major antioxidant enzymes involved in ROS detoxification.

It was revealed that the activities of catalase and SOD (antioxidant enzymes directly involved in ROS detoxification) increased several times in the cells of *Y. lipolytica* in the course of adaptation to ethanol.

The activities of the enzymes maintaining the cell's redox potential (glucose-6-phosphate dehydrogenase providing the level of reducing equivalents and glutathione reductase maintaining the pool of reduced glutathione) were noted to increase.

Besides, the activity of NAD^+-dependent alcohol dehydrogenase was shown to increase under these conditions more than 20-fold.

The increase of all the above enzyme activities was observed at the transition to the stationary growth phase, which is in agreement with the data on survival under stress conditions.

Table 1. Respiratory activity of *Y. lipolytica* under ethanol stress

Experimental conditions, growth phase	Respiration, nmol O_2 min^{-1} mg^{-1} dry cells	Respiration inhibition, %			Alternative oxidase activity, nmol O_2 min^{-1} mg^{-1} dry cells
		KCN, 1 mM	Antimycin A, 5 μM	KCN (1 mM) + BHA (5 mM)	
Exponential, without pretreatment	15.8	98	98	100	0
Stationary, without pretreatment	10.9	+190*	+160*	96	32
Exponential, pretreatment with ethanol (1%, 60 min)	16.0	65	68	95	6.0

Notes: * Symbol "+" indicates the stimulating effect of the inhibitor.
** BHA is benzhydroxaomic acid, the inhibitor of alternative oxidase.

Table 2. The activities of antioxidant enzymes of *Y. lipolytica* under ethanol stress

Culture growth phase and conditions	Activity, μmol min^{-1} mg^{-1} protein				
	NAD^+-dependent alcohol dehydrogenase	Catalase	SOD	Glucose-6-phosphate dehydrogenase	Glutathione reductase
Exponential, without pretreatment	4.2 ± 1.2	28.0 ± 1.3	4.2 ± 0.45	73.0 ± 2.3	24.0 ± 2.1
Exponential, pretreatment with ethanol (1%, 60 min)	110 ± 11.0	90.0 ± 13.6	7.5 ± 0.5	185.2 ± 15.6	56.3 ± 7.6
Stationary, without pretreatment	120 ± 10.0	75 ± 4.3	8.4 ± 1.1	103.0 ± 4.0	69.0 ± 1.3

Comparison of the results presented in this work and the literature data suggests the following sequence of events, which occur in a cell in response to the effect of ethanol. Addition of ethanol leads to impaired permeability of both the cytoplasmic and mitochondrial membrane [5–7], deceleration of electron transfer via the respiratory chain (Fig. 6), activation of ROS formation [1] and, consequently, deenergization of the cell [5–7, 11]. As a result, apart from the induction of protective genes, there is a decrease in the level of cAMP (Fig. 5) and activation of protective genes controlling the biosynthesis of antioxidant enzymes: catalase, SOD, glutathione reductase, and alternative oxidase [1, 14, 17, 21].

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