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EXPERIMENTAL ARTICLES

Tolerance of the Yeast *Yarrowia lipolytica* to Oxidative Stress

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Abstract—The adaptive response of the yeast *Yarrowia lipolytica* to the oxidative stress induced by the oxidants hydrogen peroxide, menadione, and juglone has been studied. H_2O_2, menadione, and juglone completely inhibited yeast growth at concentrations higher than 120, 0.5, and 0.03 mM, respectively. The stationary-phase yeast cells were found to be more resistant to the oxidants than the exponential-phase cells. The 60-min pretreatment of logarithmic-phase cells with nonlethal concentrations of H_2O_2 (0.3 mM), menadione (0.05 mM), and juglone (0.005 mM) made the cells more resistant to high concentrations of these oxidants. The adaptation of yeast cells to H_2O_2, menadione, and juglone was associated with an increase in the activity of cellular catalase, superoxide dismutase, glucose-6-phosphate dehydrogenase, and glutathione reductase, the main enzymes involved in cell defense against oxidative stress.

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Key words: yeast, *Yarrowia lipolytica*, oxidative stress, hydrogen peroxide, menadione, juglone, survival, adaptation mechanism, catalase, superoxide dismutase, glucose-6-phosphate dehydrogenase, glutathione reductase.

It is known that reactive oxygen species (ROSs), including hydrogen peroxide (H_2O_2), the superoxide radical (O_2^-), and the hydroxyl ion (OH^-), are toxic to microbial cells. The toxicity of these species is determined by their ability to oxidize lipids [1], proteins [2], and DNA [3].

In response to the action of ROSs, many organisms activate the synthesis of antioxidant enzymes (catalase, superoxide dismutase, glucose- 6-phosphate dehydrogenase, and others) and the production of reduced metabolites capable of detoxifying the ROSs (reduced glutathione, NAD(P)H, etc.) [4, 5]. The mechanisms of yeast adaptation to oxidative stress are as yet poorly understood and attract much research interest.

The aim of the present investigation was to study the survival of the yeast *Yarrowia lipolytica* under oxidative stress, with special emphasis on stress-induced changes in the catalase, superoxide dismutase, glucose-6-phosphate dehydrogenase, and glutathione reductase activities in yeast cells.

MATERIALS AND METHODS

The yeast *Yarrowia lipolytica* VKM Y-2378 (= *Y. lipolytica* Y-155) used in this study was obtained from the All-Russia Collection of Microorganisms (VKM). The yeast was cultivated at 29°C on a shaker (200 rpm) in 750-ml flasks with 100 ml of Reader medium [6] containing 1% glucose as the source of carbon and energy. Growth was monitored by measuring the culture turbidity at 540 nm.

To evaluate yeast survival under oxidative stress, cells of the exponential-phase (10–12 h of growth) and the stationary-phase (24 h of growth) culture were harvested by centrifugation, washed with distilled water, resuspended in 50 mM phosphate buffer (pH 7.2) and incubated in the presence of H_2O_2 or the superoxide-generating substances menadione (2-methyl-1,4-naphthoquinone) and juglone (5-hydroxy-1,4-naphthoquinone). The oxidant concentrations and the incubation time were varied.

To assess cell survival, aliquots of a cell suspension were taken at 20-min intervals, appropriately diluted with the growth medium, and plated on malt extract agar. The number of colonies grown on the agar plates was determined after incubating them at 29°C for 48–72 h. The data presented are the average results of triplicate experiments.

To prepare extracts for the enzyme assay, cells were harvested by centrifugation, washed twice with distilled water, resuspended in 50 mM phosphate buffer containing 0.5 mM phenylmethanesulfonyl fluoride (PMSF, an inhibitor of proteases), and disrupted in a French press. The homogenate was centrifuged at 105000 *g* for 60 min. The pellet was discarded, and the supernatant was used for the enzyme assay.

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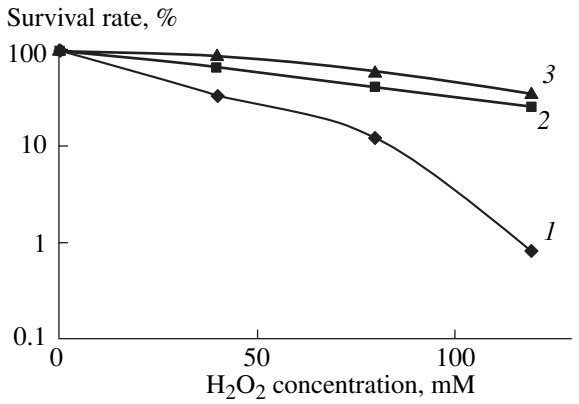

Fig. 1. The survival rates of *Y. lipolytica* cells at different H_2O_2 concentrations: (*1*) untreated exponential-phase cells, (*2*) untreated stationary-phase cells, and (*3*) exponential-phase cells pretreated with 0.3 mM H_2O_2 for 60 min.

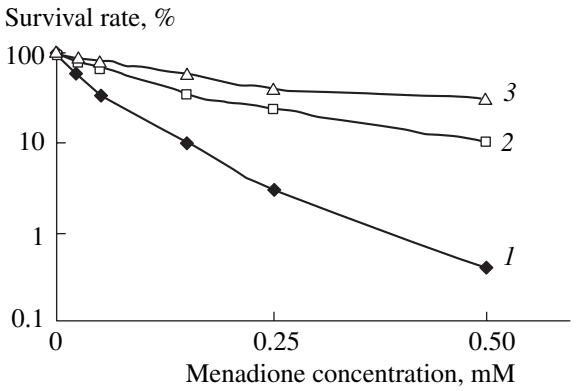

Fig. 2. The survival rates of *Y. lipolytica* cells at different menadione concentrations: (*1*) untreated exponential-phase cells, (*2*) untreated stationary-phase cells, and (*3*) exponential-phase cells pretreated with 0.05 mM menadione for 60 min.

The activity of catalase was determined from the decrease in the H_2O_2 concentration, which was estimated by measuring the optical density of the reaction mixture at 246 nm [7].

The activity of superoxide dismutase (SOD) was determined by measuring the degree of inhibition of the reduction of 10 μM cytochrome *c* by the superoxide radical in 50 mM Tris–HCl (pH 7.5) containing 0.5 mM xanthine and 0.5 U xanthine oxidase [8].

Glucose-6-phosphate dehydrogenase was assayed by measuring the reduction of $NADP^+$ at 340 nm [9].

Glutathione reductase was assayed by measuring the oxidation of NADPH at 340 nm in the presence of oxidized glutathione [9].

Protein was quantified with the biuret reagent.

Spectral measurements were carried out using a Shimadzu UV-160 spectrophotometer (Japan).

The reagents used were a 3% solution of locally produced H_2O_2, cytochrome *c* and juglone purchased from Sigma, and PMSF, menadione, xanthine, and xanthine oxidase purchased from ICN.

RESULTS AND DISCUSSION

Figure 1 illustrates the effect of H_2O_2 on yeast survival. As can be seen from this figure, elevated concentrations of H_2O_2 diminished cell survival. The concentration of H_2O_2 equal to 120 mM was found to be lethal to exponential-phase *Y. lipolytica* cells (Fig. 1, curve *1*).

Various yeasts show different sensitivity to H_2O_2. For example, the exposure of *Saccharomyces cerevisiae* cells to 2 mM H_2O_2 for 60 min or to 10 mM H_2O_2 for 15 min leaves only 1% of exposed cells viable [10]. On the other hand, a noticeable decrease in the survival rate of *Schizosaccharomyces pombe* cells was observed only at H_2O_2 concentration equal to 300 mM [11]. The sensitivity of *Y. lipolytica* cells to H_2O_2 is the closest to that of *Candida albicans* cells (total inhibition of growth at the concentration of H_2O_2 equal to ~100 mM) [12].

As shown by Lee et al. on *Schiz. pombe* cells [13], the survival of yeast cells in the presence of a lethal concentration of an oxidant can be improved by preincubating these cells in the presence of a nonlethal concentration of the oxidant. In our experiments, the pretreatment of the exponential-phase *Y. lipolytica* cells with 0.3 mM H_2O_2 for 60 min augmented the survival rate of these cells in the presence of 120 mM H_2O_2 to 35% (Fig. 1, curve *3*). For further experiments, we selected 0.3 mM as a nonlethal concentration of H_2O_2 to *Y. lipolytica* cells. This H_2O_2 concentration leaves 90% of the exponential-phase *Y. lipolytica* cells viable (Fig. 1, curve *1*). The optimal time of cell pretreatment with H_2O_2 was found to be 60 min.

Juglone and menadione can induce oxidative stress in yeast cells by generating superoxide radicals in these cells [14]. The survival of *Y. lipolytica* cells in the presence of menadione and juglone is shown in Figs. 2 and 3. At concentrations of, respectively, 0.5 and 0.03 mM, menadione and juglone leave less than 1% of the exponential-phase cells viable (Figs. 2 and 3, curves *1*). Therefore, these concentrations of menadione and juglone are lethal to *Y. lipolytica* cells.

As for the sensitivity of other yeasts to menadione and juglone, the lethal concentrations of menadione (causing 99% cell death or 1% cell survival) to *S. cerevisiae* and *Schiz. pombe* are 6 and 1 mM, respectively [15, 13]. The lethal concentrations of another superoxide-generating agent, plumbagin (2-methyl-5-hydroxynaphthoquinone), to *C. albicans* and *S. cerevisiae* cells are 0.6 and 0.06 mM, respectively [12, 15]. Based on these data, Jamieson suggested that the aerobic pathogenic yeast *C. albicans* is more tolerant to oxidants than the yeast *S. cerevisiae* [15].

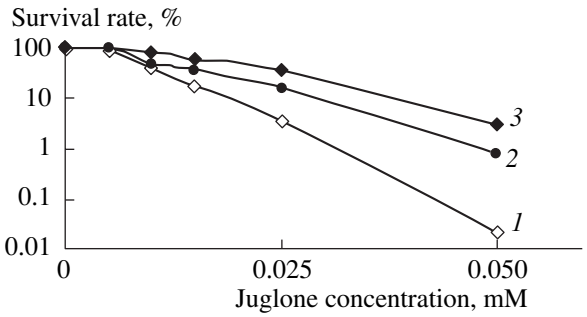

Fig. 3. The survival rates of *Y. lipolytica* cells at different juglone concentrations: (*1*) untreated exponential-phase cells, (*2*) untreated stationary-phase cells, and (*3*) exponential-phase cells pretreated with 0.005 mM juglone for 60 min.

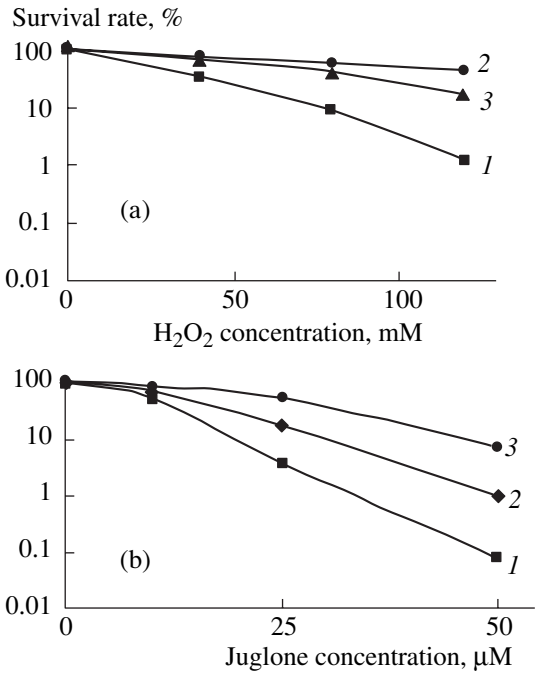

Fig. 4. The survival rates of the exponential-phase *Y. lipolytica* cells at different concentrations of (a) H_2O_2 and (b) juglone: (*1*) untreated cells, (*2*) cells pretreated with 5 μM juglone for 60 min, and (*3*) cells pretreated with 0.3 mM H_2O_2 for 60 min.

In our experiments, the pretreatment of the exponential-phase *Y. lipolytica* cells to nonlethal concentrations of menadione (0.05 mM) and juglone (0.005 mM) enhanced cell tolerance to high concentrations of these oxidants (Figs. 2 and 3, curves *3*).

According to the data available in the literature [10, 11] and those presented in this study (Figs. 1–3, curves *2*), stationary-phase yeast cells are more resistant to oxidants than unadapted exponential-phase cells (Figs. 1–3, curves *1*). This fact can be explained by the activation of the regulatory mechanisms responsible for cell resistance to oxidative stress in the stationary growth phase or by a diminished permeability of the stationary-phase yeast cells to H_2O_2 and other oxidants. As shown by Sousa-Lopes et al. [16], the permeability of stationary-phase cells of *S. cerevisiae* to H_2O_2 is five times lower than that of exponential-phase yeast cells, which may be due to the enhanced synthesis of ergosterol responsible for the permeability of the cytoplasmic membrane. Mutants with impaired synthesis of ergosterol show a higher cell permeability to H_2O_2 and a lower cell survival rate [16].

The study of the ability of *Y. lipolytica* to improve its survival in response to exposure to nonlethal concentrations of various stressful agents showed that the pretreatment of exponential-phase *Y. lipolytica* cells with 0.005 mM juglone (Fig. 4a, curve *2*) enhanced their resistance to high concentrations of H_2O_2 and, vice versa, the pretreatment of these cells with 0.3 mM H_2O_2 enhanced their resistance to high concentrations of juglone (Fig. 4b, curve *3*). These data are in agreement with earlier studies on *C. albicans* [13] and *Fusarium decemcellulare* [5]. Therefore, the adaptive mechanisms of various microorganisms to oxidative stress are similar.

It should be noted that the resistance of the H_2O_2-pretreated exponential-phase *Y. lipolytica* cells to juglone was somewhat higher than that of the juglone-pretreated cells, and the resistance of the juglone- pretreated cells to H_2O_2 was slightly higher than that of the H_2O_2-pretreated cells. This finding suggests that the cross-resistance of yeast cells has a high adaptive potential. Similar results were obtained for stationary-phase *Y. lipolytica* cells (data not shown).

By contrast, the pretreatment of *S. cerevisiae* cells with nonlethal concentrations of menadione does not augment their resistance to H_2O_2 and the pretreatment with H_2O_2 does not increase cell resistance to menadione [15]. This fact suggests that yeasts from different taxonomic groups differ in their adaptive mechanisms.

To gain insight into the mechanisms responsible for the increased resistance of yeast cells to oxidants, we measured the activities of four antioxidant enzymes in the exponential-phase cells of *Y. lipolytica* before and after treating them with the oxidants (see table). As can be seen from the table, the activity of catalase rose by 3.3 and 4.2 times, respectively, when yeast cells were pretreated with 0.3 mM H_2O_2 and 0.05 mM menadione, to reach 93 and 120 μmol/(min mg protein). These values of catalase activity are comparable to that reported for *Schiz. pombe* cells adapted to oxidative stress [13].

The pretreatment of *Y. lipolytica* cells with menadione and H_2O_2 increased the SOD activity by 5 and 1.5 times, respectively. The level of SOD in stationary-phase *Y. lipolytica* cells was twice as high as in exponential-phase cells. In general, the initial level of SOD activity in *Y. lipolytica* cells is lower than in *Schiz. pombe* [13] and *C. albicans* [12], which may explain the higher sensitivity of *Y. lipolytica* cells to superoxide-generating agents (see above).

Activities of antioxidant enzymes in *Y. lipolytica* cells from different growth phases and the effect of cell pretreatment with hydrogen peroxide and menadione

Cells	Growth phase	Activity, μmol/(min mg protein)			
		Catalase	SOD	Glucose-6-phosphate dehydrogenase	Glutathione reductase
Untreated	Exponential	28.0 ± 3.3	4.2 ± 4.5	73.0 ± 6.8	24.1 ± 2.6
Untreated	Stationary	75.0 ± 8.3	8.4 ± 9.1	103.0 ± 9.4	69.2 ± 5.3
Pretreated with 0.3 mM H_2O_2	Exponential	93.0 ± 10.1	6.5 ± 6.7	116.0 ± 10.9	58.1 ± 6.4
Pretreated with 0.05 mM juglone	Exponential	120.0 ± 13.6	21.6 ± 2.3	138.2 ± 13.6	66.3 ± 7.6

Thus, the increase in the survival rate of the exponential-phase yeast cells in response to their pretreatment with low doses of oxidants may be accounted for by the activation of various adaptive mechanisms, including an increase in catalase and SOD activities.

Minard and McAlister-Henn [17] reported on the antioxidant function of NAD(P)H in yeast cells. With this in mind, we measured the levels of glucose-6-phosphate dehydrogenase and glutathione reductase in *Y. lipolytica* cells (see table) and found that these levels increased in response to the transition of these cells to the stationary growth phase or to their pretreatment with low doses of antioxidants. In particular, glutathione reductase activity in stationary-phase cells was found to be more than two times higher than in the exponential-phase cells. The pretreatment of exponential-phase *Y. lipolytica* cells with the oxidants enhanced their glucose-6-phosphate dehydrogenase activity by 1.5–2 times (table), which is in agreement with the data obtained by Lee et al. for the yeast *Schiz. pombe* [13]. Of interest is the fact that the normal level of glucose-6- phosphate dehydrogenase in the yeast *Y. lipolytica* is higher than in *C. albicans* [12] and *Schiz. pombe* [13].

To conclude, the adaptation of yeast cells to oxidative stress activates the enzymes that are involved in the detoxification of ROSs.

The molecular mechanism responsible for the adaptive response of yeasts to oxidative stress is as yet unknown in depth. Taking into account that a particular stress factor can induce tolerance to other stress factors, we can suggest the existence of a common signal that triggers defense mechanisms in microbial cells.

According to Sousa-Lopes et al. [16], the stationary-phase cells of yeasts and other microorganisms are more resistant to a variety of stress factors, including heat shock, than the exponential-phase cells. The pretreatment of yeast cells with 0.3 mM H_2O_2 makes them resistant to heat shock, high ethanol concentrations, and other stresses [18]. Conversely, the preliminary exposure of yeast cells to heat shock makes them resistant to hydrogen peroxide [15].

The tolerance of yeasts to stresses can be accounted for by the elimination of the negative regulatory effect (when the RAS–cAMP–PKA pathway is functioning) on the expression of the stress genes, as well as by the enhanced expression of the antioxidant enzyme genes [10, 18].

In yeasts, oxidative and other stresses induce the transcription of the catalase gene mediated by specific regulatory sequences (the so-called stress response elements) [3, 10, 19, 20]. Depending on the conditions, gene transcription is regulated by cAMP-dependent or cAMP-independent mechanisms [21]. This fact suggests that the factors responsible for changes in the cellular pool of cAMP may induce adaptive responses in cells. This suggestion can be verified by further studies into the effect of various stress factors on the level of cAMP in yeast cells.

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