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EXPERIMENTAL ARTICLES

Synthesis and Localization of L-Lactate Oxidase in Yeasts Yarrowia Lipolytica

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Abstract— Conditions for L-lactate oxidase synthesis by the yeast *Yarrowia lipolytica* were investigated. The enzyme was found to be synthesized during growth on L-lactate in the exponential growth phase. L-lactate oxidase synthesis was also observed on glucose after adaptation to stress conditions (oxidative or thermal stress) during the stationary growth phase after glucose consumption. The cells grown on L-lactate exhibited high levels of antioxidant enzymes (catalase, superoxide dismutase, glucose-6-phosphate dehydrogenase, and glutathione reductase), which exceeded those of glucose-grown cells. Ultrastructurally, L-lactate-grown cells and the cells grown on glucose and adapted to various stress conditions were also found to be similar, with increased mitochondria, elevated number and size of peroxisomes, and formation of lipid and polyphosphate inclusions. In order to determine the intracellular localization of L-lactate oxidase, the cells were disintegrated by the lytic enzyme complex from *Helix pomatia*. Centrifugation of the homogenate in Percoll gradient resulted in the isolation of purified fractions of the native mitochondria and peroxisomes. L-lactate oxidase was shown to be localized in peroxisomes.

Keywords: *Yarrowia lipolytica*, stress, antioxidant enzymes, L-lactate oxidase, localization, mitochondria, peroxisomes

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L-lactate oxidase (EC 1.1.3.15) is a flavin enzyme catalyzing the oxidation of L-lactate to pyruvate, with oxygen reduction to hydrogen peroxide. This enzyme has been found in bacteria *Aerococcus viridans* [1], *Streptococcus faecalis* [2], *Pediococcus* sp. [3], and *Lactococus lactis* [4], as well as in the fungi *Geotricum candidum* [5] and *Yarrowia lipolytica* [6].

Lactate oxidase is widely used in various fields, such as medicine, food industry, etc. For example, this enzyme is used to determine the content of lactate in human physiological fluids (blood, cerebrospinal fluid, etc.) in different pathological states. The level of lactic acid is an important clinical diagnostic indicator of hypoxia, lactic acidosis, state of shock, acute myocardial infarction, and other diseases associated with inadequate oxygen supply of oxygen to the tissues.

Monitoring the level of this metabolite in blood or sweat of athletes is an important instrument of workout session control, which is used to assess the efficiency of gymnastics equipment and exercise regimens [7].

The content of lactate is also an important indicator for wine, dairy, and meat production. Lactate oxidase is a basis of biosensors [8] designed for medicine, wine-making, and the food industry.

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Previously, it has been shown that L-lactate oxidase appears in *Y. lipolytica* cells during their growth on L-lactate as the only carbon and energy source [6]. When the yeast grows on L-lactate, the primary reaction of its oxidation is coupled with the formation of hydrogen peroxide, which is toxic for the cells. Consequently, there must be a mechanism of hydrogen peroxide neutralization. For example, it may be degraded by catalase localized in peroxisomes, while the number of peroxisomes and the content of enzymes in these organelles may vary depending on external conditions [9].

The goal of the present work was to study the conditions of L-lactate oxidase synthesis and subcellular localization of the enzyme in the yeast *Y. lipolytica*.

MATERIALS AND METHODS

The subject of research was the yeast *Y. lipolytica* VKM Y-2378 obtained from the All-Russian Collection of Microorganisms, 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 Reader medium [10] with glucose (1%) or L-lactate (2%) on a shaker (200 rpm). Yeast growth was assessed by optical density (λ = 540 nm). The cells of the exponential or stationary

growth phases were washed twice with sterile distilled water and suspended in 50 mM Tris-phosphate buffer, pH 7.2.

The yeast growing on glucose was adapted to stress conditions by exposure to nonlethal doses of oxidants or "heat habituation" [10, 11]. For this purpose, the exponential-phase cells (10–12 h) were incubated for 60 min either at 37°C (heat shock) or in the growth medium with 0.5 mM H_2O_2 at 22°C (oxidative stress).

The protoplasts of the yeast grown on L-lactate were obtained to determine the localization of L-lactate oxidase. The cells were washed with 0.9% NaCl and resuspended in the medium containing 0.9 M sucrose, 25 mM Tris–phosphate buffer (pH 7.2), 0.5 mM EDTA (medium *A*), and lyophilized snail gastric juice (0.5 g/10 g wet cells) [12]. Lyophilized snail gastric juice was prepurified in a Sephadex G-25 column to remove heavy metal ions. The cells (100–150 mL of the suspension) were incubated with snail gastric juice (60–90 min) in flasks (750 mL) on a shaker (200 rpm) until 80–90% of the cells turned to protoplasts. The protoplasts were washed three times with the medium *A*, followed by centrifugation at 7000 *g* for 20 min, and then homogenized in a Potter homogenizer in the medium containing 0.5 M mannitol, 25 mM Tris–phosphate buffer (pH 7.2), and 0.5 mM EDTA (medium *B*). The remaining protoplasts and nuclei were precipitated by sequential centrifugation at 1000 and 2000 *g* for 15 min. Mitochondria were precipitated from the supernatant by centrifugation at 5000 *g* for 15 min. The pellet containing mainly heavy mitochondria was resuspended in medium *B* (20 mL). Then the supernatant was centrifuged at 7000 *g* for 15 min to remove light mitochondria. The pellet was discarded, and the supernatant was centrifuged at 20000 *g* for 20 min. The resultant pellet containing mainly peroxisomes and an admixture of small mitochondria was resuspended in medium *B* with mannitol (20 mL). The supernatant was centrifuged at 100000 *g* for 60 min. As a result, the cytosol fraction and the fraction of light membranes were obtained in the pellet.

The mitochondrial and microsomal fractions were resuspended in medium *B* (180 mL) containing Percoll (30%). The suspension was then centrifuged at 30000 *g* for 30 min (4°C) in 40-mL test tubes in a Vti-50 vertical rotor (Beckman L5-75). The gradient profile was determined by the distribution of stained marker granules of known density. Aliquots (3 mL) were taken from the bottom of centrifuge tubes using Recovery system (Beckman).

Cell-free extracts were obtained by washing the cells twice with distilled water and resuspending in 50 mM Tris-phosphate buffer (pH 7.0) with 0.5 mM phenylmethylsulfonyl fluoride (a protease inhibitor), followed by disruption in a French press. The homogenate was centrifuged at 105000 *g* for 60 min. The pellet was discarded, and the supernatant was used to determine the activities of antioxidant enzymes and of lactate oxidase.

Catalase activity was assayed spectrophotometrically by the change in H_2O_2 absorption (E_{240} = 0.32 mM^{-1} cm^{-1}) [13].

Superoxide dismutase (SOD) activity was assayed by the inhibition of reduction of 10 μM cytochrome *c* in the presence of 0.5 mM xanthine and 0.5 U xanthine oxidase (λ = 550 nm) [14]. The enzyme amount that caused 50% inhibition of the rate of cytochrome *c* reduction was taken as a unit of activity.

Isocitrate lyase and glucose-6-phosphate dehydrogenase activities were assayed according to Dixon and Kornberg [15].

Glutathione reductase activity was measured by the decrease in NADPH in the presence of oxidized glutathione (E_{340} = 6.22 mM^{-1} cm^{-1}) [16].

The concentrations of cytochromes $a + a_3$ were determined by differential absorption spectra (E_{605} = 14 mM^{-1} cm^{-1}) [17].

Cytochrome oxidase activity was measured by the rate of oxidation of reduced cytochrome *c* (λ = 550 nm) [17].

Lactate oxidase activity of the soluble fraction was recorded spectrophotometrically by the rate of hydrogen peroxide production in 20 mM Tris-phosphate buffer (pH 8.0) in the presence of *o*-dianisidine (0.2 mM), peroxidase (5 μg/mL) and L-lactate (2 mM) (E_{436} = 8.3 mM^{-1} cm^{-1}).

Lactate oxidase activities of the membrane fraction, homogenate, and intact cells were determined by the rate of oxygen consumption using a Teflon film-covered Clark-type platinum electrode in the presence of 5 mM L-lactate (nmol O_2 min^{-1} mg^{-1} of dry cells or μmol O_2 min^{-1} mg^{-1} of protein).

Protein content was assayed using the biuret reagent.

Cell ultrastructure was studied by the Reynolds method [19]. Ultrathin sections were examined in a JEM-100B electron microscope (JEOL, Japan) at 80 kV [20].

The following reagents were used in the work: H_2O_2 (3%) (Biosintez, St. Petersburg, Russia); cytochrome *c*, xanthine, xanthine oxidase, phenylmethylsulfonyl fluoride (ICN); L-lactate, mannitol, EDTA, *o*-dianisidine, and peroxidase (Sigma).

The average results of three replicates in three experimental series are presented, with standard deviations calculated for the probability $p > 0.95$.

RESULTS AND DISCUSSION

It has been shown previously that *Y. lipolytica* cells synthesize lactate oxidase when growing on L-lactate as the only carbon and energy source [16].

In this work we demonstrated the ability to synthesize lactate oxidase by its cells grown on glucose. This

Table 1. Conditions for L-lactate oxidase synthesis by the yeast *Yarrowia lipolytica*

Substrate, growth phase	Glucose exponential	Glucose, stationary	Glucose, exponential, oxidative stress, (H_2O_2)	Glucose, exponential, thermal stress, (37°C)	L-lactate
L-lactate oxidase activity, nmol O_2 min^{-1} mg^{-1} dry cells	0	20.1 ± 1.4	35.4 ± 2.0	25.2 ± 1.4	120.0 ± 10.2

Table 2. Activities of L-lactate oxidase and antioxidant enzymes (μmol min^{-1} mg^{-1} protein) in the yeast *Yarrowia lipolytica*

Conditions	L-lactate oxidase	Catalase	Glucose-6-phosphate dehydrogenase	Superoxide dismutase	Glutathione reductase
Exponential growth phase on lactate	40.2 ± 3.2	185.0 ± 11.0	148.0 ± 8.6	18.5 ± 0.6	76.3 ± 7.6
Exponential growth phase on glucose	0	28.0 ± 1.3	73.0 ± 2.3	4.2 ± 0.5	24.0 ± 2.6

ability was observed after the yeast had passed to the stationary growth phase, at glucose depletion in the medium (Table 1). It should be noted that the cells growing on L-lactate, in contrast to glucose-grown cells, synthesized lactate oxidase during the exponential growth phase; lactate oxidase biosynthesis was not observed in the stationary growth phase.

It was also shown that lactate oxidase could be synthesized by the exponential-phase yeast growing on glucose after the adaptation to stress conditions, e.g., after exposure to oxidants (oxidative stress) or elevated temperature (heat stress) (Table 1).

The results presented in Table 2 show that the cells growing on L-lactate had higher levels of antioxidant enzymes (catalase, SOD, glucose-6-phosphate dehydrogenase, and glutathione reductase) than the cells growing on glucose.

Enhanced activity of the antioxidant systems in *Y. lipolytica* has been shown previously as a protective response during yeast adaptation to various stresses or upon transition to the stationary growth phase after glucose depletion [10, 11].

The high antioxidant activity during growth on L-lactate can be accounted for by the fact that the primary reaction of lactate oxidation is coupled with hydrogen peroxide production, which creates oxidative stress conditions. The above data suggest that the cells synthesize lactate oxidase in response to stress impacts.

Ultrastructure of the cells growing on L-lactate has been comparatively studied in this respect. Figure 1 shows more numerous and larger peroxisomes in the cells growing on L-lactate (Fig. 1b) compared to the cells growing on glucose (Fig. 1a). Mitochondria under these conditions were larger and had a denser matrix with the greater number of cristae. Moreover, polyphosphate and lipid inclusions were found inside the cytoplasm of L-lactate-grown cells (Fig. 1b).

These ultrastructural features of the yeast cells growing on L-lactate were revealed previously in glucose-grown *Y. lipolytica* cells and after the adaptation to various stresses [20]. It should be added that the cell wall of L-lactate-grown yeasts, in contrast to the cells growing on glucose, was shown to have structurally modified regions resembling the shape of a channel without any distinct limiting contour.

Protoplasts, as well as mitochondrial and peroxisomal fractions, were obtained in order to determine the localization of lactate oxidase in yeast cells growing on lactate. Figures 2 and 3 present the data on the distribution of enzyme activities among the fractions after centrifugation in Percoll. It can be seen (Fig. 2) that the marker enzymes of peroxisomes, i.e., catalase (curve *2*) and peroxidase (curve *3*), were found in the upper zone of Percoll and showed the maximum activ-

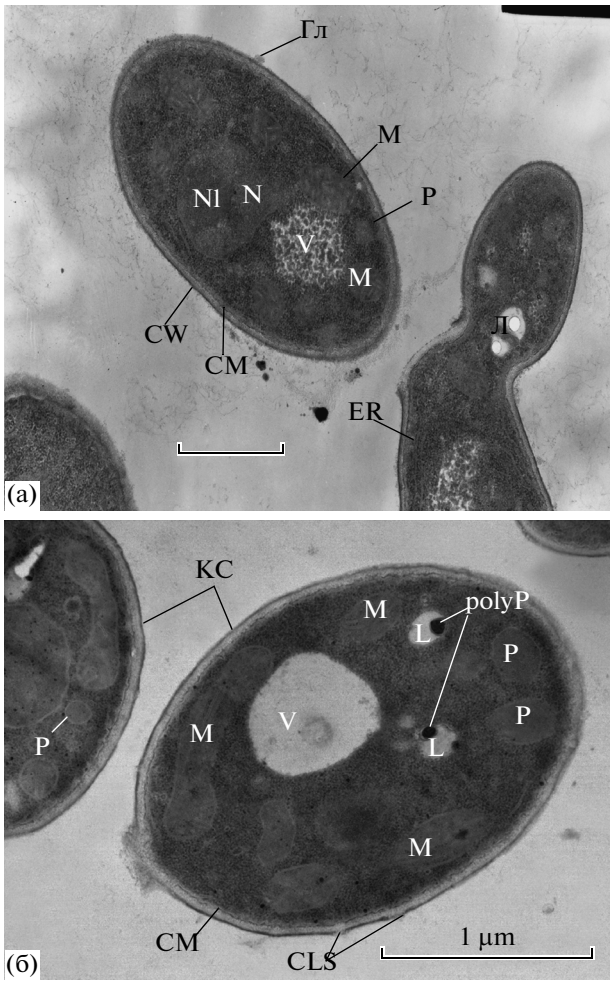

Fig. 1. Ultrathin section of *Yarrowia lipolytica* cells from the exponential growth phase on glucose (a) and L-lactate (b). Scale bar: 1 μm. Designations: N, nucleus; Nl, nucleolus; CW, cell wall; CM, cytoplasmic membrane; ER, endoplasmic reticulum; M, mitochondrion; P, peroxisome; V, vacuole; L, lipids; polyP, polyphosphates; CLS, channel-like structures.

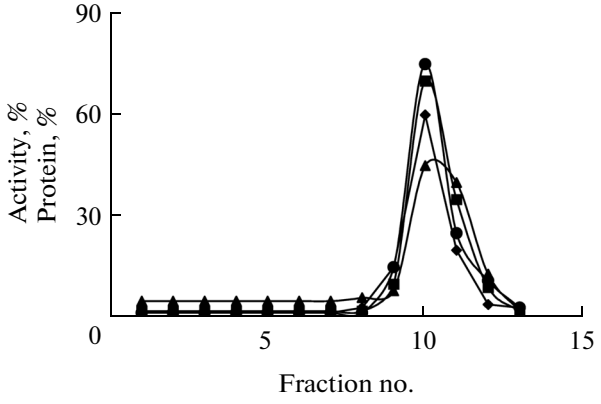

Fig. 2. Enzyme activities in peroxisomal fractions after centrifugation in Percoll. Catalase (◆), isocitrate lyase (▲), L-lactate oxidase (■), protein content (●).

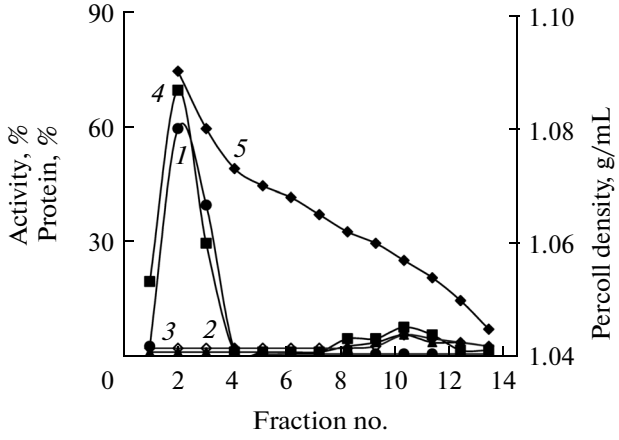

Fig. 3. Enzyme activities in mitochondrial fractions after centrifugation in Percoll. Cytochrome *c*-oxidase (*1*), L-lactate oxidase (*2*), isocitrate lyase (*3*), protein content (*4*); Percoll density (*5*).

ity in fractions 9–11 (density 1.04–1.06). Lactate oxidase activity (curve *1*) was also expressed in fractions 9–11, which is indicative of its localization in the peroxisomal fraction of the cells. The major protein peak (curve *4*) was also revealed in the peroxisomal fraction.

Figure 3 shows that the activity of the marker enzyme of mitochondria, cytochrome oxidase (curve *5*), after centrifugation of the fraction in Percoll was expressed in the lower zone of Percoll (fractions 2 and 3) with a density of 1.07–1.08. It should be noted that less than 5% isocitrate lyase activity was found in the upper zone of Percoll (curve *3*, fraction 10), indicating an insignificant admixture of the peroxisomal fraction in the mitochondria.

Table 3 summarizes the data on the distribution of enzymatic activities among the fractions with due consideration of protein content.

Thus, the studies of intracellular localization of lactate oxidase revealed that this enzyme in the yeast *Y. lipolytica* was located in peroxisomes (85%), together with catalase and isocitrate lyase, the peroxisomal marker enzymes.

As a result of the studies, the patterns of lactate oxidase synthesis by the yeast *Y. lipolytica* were determined. It was shown that lactate oxidase synthesis during growth on L-lactate occurred already in the exponential phase (with no enzyme synthesis observed during the stationary growth phase). The cells growing on L-lactate showed high activities of the antioxidant enzymes involved in detoxification of ROS (reactive oxygen species) formed at the first stage of lactate oxidation. It was shown that the ultrastructure of the cells growing on L-lactate was analogous to that of glucose-

Table 3. Distribution of enzyme activities (μmol min^{-1} mg^{-1} protein) by the fractions of *Yarrowia lipolytica* cells (growth on lactate)

Enzyme	Homogenate	Soluble fraction (65%* protein)	Mitochondria (22%* protein)	Peroxisomes (11%* protein)	Total activity of fractions, %
Cytochrome *c*-oxidase	0.18	0	0.8 (98%)	0	
Catalase	0.60	0.13 (10%**)	0.02 (5%**)	1.25 (81%**)	96
Isocitrate lyase	0.14	0.05 (3%**)	0.015 (3%**)	0.65 (91%**)	97
L-lactate oxidase	0.20	0.04 (7%**)	0.01 (5%**)	0.73 (85%**)	97
Cytochrome $a + a_3$	0.02	0	0.17 (98%)	0.004 (2%)	100

* Protein content relative to the homogenate.

** Activity relative to the homogenate.

grown cells adapted to stress conditions. Lactate oxidase was shown to be localized in cell peroxisomes.

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