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EXPERIMENTAL ARTICLES

Production of Extracellular H_2O_2 and L-Lysine-α-Oxidase during Bulk Growth of the Fungus *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D under Salt Stress

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Abstract—In this article, we report on Some physiological aspects of the synthesis of extracellular L-lysine-α-oxidase (LO) by the fungus *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D under salt stress conditions and discuss the possible role of this enzyme for the producer. It has been shown that The synthesis of extracellular LO and proteolytic enzymes is induced in the fungus *T.* cf. *aureoviride* Rifai VKM F-4268D during submerged cultivation on wheat bran under salt stress. It has been shown that LO biosynthesis is accompanied by H_2O_2 accumulation in the growth medium. It seems that the extracellular LO synthesis followed by hydrogen peroxide production under stress conditions provides an adaptive advantage for the producer fungus in its competition with other organisms.

Keywords: L-lysine-α-oxidase, biosynthesis, proteases, *Trichoderma*, hydrogen peroxide

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L- and D-amino acid oxidases are widespread in nature and have been found in animals [1, 2] and microorganisms, including yeasts [3], fungi [4–9], and bacteria [10–14]. In contrast to animal amino acid oxidases, the oxidases of microbial amino acids are characterized by several essential peculiar features as regards their properties and conditions of biosynthesis.

First, the microbial amino acid oxidases have narrow substrate specificity: D-aspartate oxidase [3], L-glutamate oxidase [12], L-phenylalanine oxidase [13], and L-lysine oxidase [14] oxidize mainly the respective amino acids and show a very weak activity against other amino acids.

Second, some of them, including L-lysine-α-oxidase (LO) from the *Trichoderma* fungi, are extracellular enzymes excreted from the cells during the growth of the producer [7, 9, 14].

The fact of extracellular LO accumulation brings up the logical question about the physiological role of this enzyme for the producer.

The study of LO preparations from the fungus *T. viride* has shown that the enzyme possesses antibacterial and antitumor activities [6, 8, 15]. At the same time, the cytotoxic and antibacterial effects of LO manifested themselves only under aerobic conditions and in the presence of L-lysine.

Analogous data were obtained also with L-lysine-ε-oxidase from the marine bacterium *Marinomonas mediterranea* [10, 11]: the antibacterial activity of this enzyme was found in the presence of oxygen and L-lysine. In the presence of catalase, the antibacterial effect was not observed. It is natural that hydrogen peroxide production was observed in all cases when L-lysine-ε-oxidase demonstrated its antibacterial effect.

Previously we have shown the synthesis of extracellular LO by the fungus *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D [9, 16]. We have assumed H_2O_2 production in the course of LO biosynthesis, which is a significant factor of competition of the fungus with other microorganisms under natural conditions.y

The objective of this study was to define some physiological aspects of LO synthesis by the fungus *T.* cf. *aureoviride* Rifai VKM F-4268D: to show the presence of hydrogen peroxide in the growth medium and to find out the factors controlling this process.

MATERIALS AND METHODS

The object of research was the fungus *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D from the All-Russian Collection of Microorganisms (VKM, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences) capable of synthesizing extracellular LO [9].

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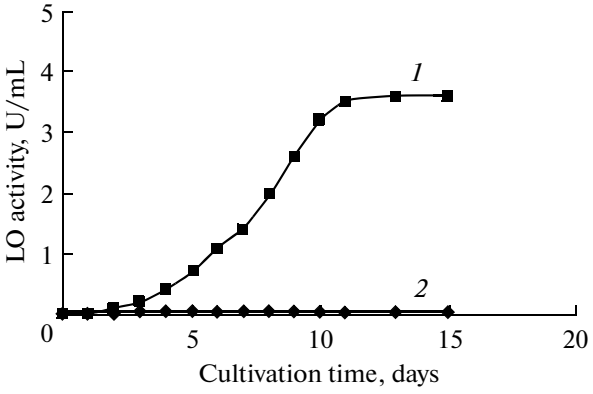

Fig. 1. The synthesis of L-lysine-α-oxidase during the growth of the fungus *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D on wheat bran in the presence (*1*) and absence (*2*) of NaCl.

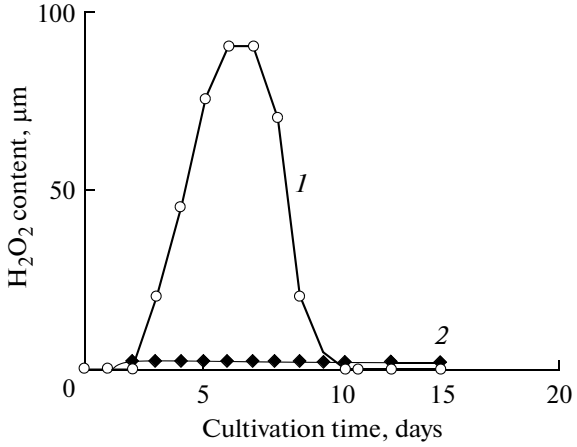

Fig. 2. The content of H_2O_2 in the cultivation medium of *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D in the presence (*1*) and absence (*2*) of NaCl.

The fungus was grown in Czapek's medium containing (g/L): KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; yeast autolysate, 0.05. Wheat bran (60 g/L) was used as a growth substrate. NaCl was added at concentrations of 2 to 8%. Cultivation was performed in 750 mL flasks containing 100 mL of the medium for 10–13 days in a shaker (220 rpm) at 29°C.

The efficiency of biosynthesis was estimated by LO accumulation in the growth medium and expressed in international units of enzyme activity per unit of the volume (U/mL).

LO activity was assayed at 20°C 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-lysine (2 mM) with a Shimadzu spectrophotometer ($E_{436} = 8.3$ mM^{-1} cm^{-1}) [9, 17]. The enzyme quantity catalyzing the oxidation of 1 μmole lysine per min was taken as a unit of activity.

Proteolytic activity in the samples was assayed with stained casein [18].

Hydrogen peroxide in the culture liquid was assayed by the change in *o*-dianisidine absorption in the presence of peroxidase in a Shimadzu spectrophotometer ($E_{436} = 11.0$ mM^{-1} cm^{-1}) [17]. The reaction mixture containing *o*-dianisidine (0.2 mM) in 25 mM Tris-phosphate buffer (pH 7.5) was added up to 1 mL to the aliquot of the culture liquid (0.1–0.2 mL). The reaction was started by adding peroxidase (up to 0.5 μg/mL). Upon color development, the sample was centrifuged for 5 min at 12000 *g*.

Free amino acids were assayed in LC2000 Biotronic amino acid analyzer (Germany). To this end, the aliquots of culture liquid were treated with perchloric acid (the final concentration of 5%).

The presented data are the averaged results of three repeats in three experimental series with calculating standard deviations for the probability $p > 0.95$.

RESULTS AND DISCUSSION

Previously it has been shown [9, 16] that LO is synthesized by the fungus *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D during cultivation in the media containing wheat bran and in the presence of inorganic nitrogen salts. It was shown that NaCl could be used instead of nitrogen salts for stimulating LO synthesis. The maximum level of LO biosynthesis was observed at a 6% NaCl concentration in the growth medium, with an ionic force of ~1M.

As Figure 1 shows, LO activity in the presence of NaCl was observed already on day 2 of cultivation and reached the maximum level (4 U/mL) on day 10–11 (curve *2*). Without the salt, the extracellular LO activity was actually absent.

It should be noted that salt concentrations previously defined as optimal for LO biosynthesis [16] also correspond to the ionic force of ~1 M. It may be supposed that the above conditions (the presence of salts) are salt stress conditions provoking LO synthesis in the fungus.

Figure 2 shows the results of H_2O_2 concentration measurement in the cultivation medium of the fungus in the presence and absence of the salt. One can see that in the presence of the salt (6%) H_2O_2 was found in the medium on day 2–3 of fungal growth, i.e., simultaneously with the beginning of LO biosynthesis. The maximum concentration was observed on day 6–7 of growth. In the absence of the salt, H_2O_2, just as LO, practically was not detected in the growth medium.

The findings suggest the dependence between LO activity and H_2O_2 production in the growth medium.

We have studied the activity of extracellular proteolytic enzymes that hydrolyzed the protein components of wheat bran and supplied amino acids, including lysine, to the growth medium. As seen in Figure 3, proteolytic activity appeared on day 2 of fungal growth

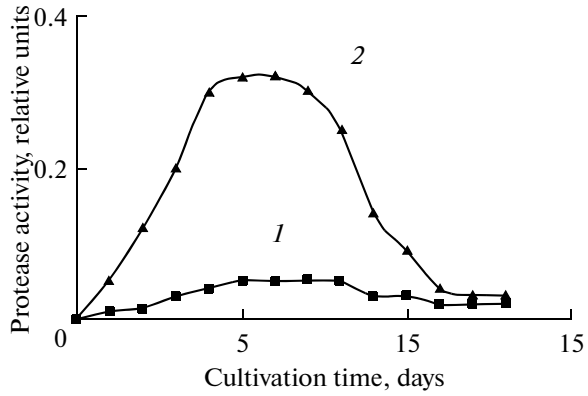

Fig. 3. The activity of proteolytic enzymes during the cultivation of *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D in the absence (*1*) and presence (*2*) of NaCl.

in the presence of the salt and reached the maximum value on day 5–7. The activity of proteolytic enzymes during fungal growth in the presence of NaCl (curve *2*) noticeably exceeded that in the absence of the salt (curve *1*).

It should be noted that the dynamics of proteolytic activity (Fig. 3) correlates with the dynamics of H_2O_2 accumulation in the growth medium (Fig. 2).

The results of amino acid analysis of the cultivation medium of LO-synthesizing fungus are presented in the table. More than 10 amino acids formed as a result of action of extracellular proteases were observed in the medium after 1-day cultivation. The maximum content of some amino acids (asparagine, alanine, threonine, and serine) was observed on day 3–5; after that, they were actively consumed during further growth of the fungus. Other amino acids (glutamine) gradually accumulated in the medium up to 33.8 mg/L at the end of cultivation.

It should be noted that L-lysine was detected only on day 1–2 of growth. The absence of lysine after 2 days of cultivation may be explained by the fact that the released lysine is quickly oxidized by extracellular LO with formation of hydrogen peroxide.

The results propose the following chain of events during bulk cultivation of the fungus on wheat bran. The synthesis of extracellular LO and proteolytic enzymes is induced in the fungus under stress conditions. Lysine released under the influence of proteases is oxidized by LO with formation of hydrogen peroxide, which accumulates in the cultivation medium.

It may be supposed that the extracellular LO producing hydrogen peroxide under stress conditions gives the producer fungus an adaptive advantage for competition with other organisms.

Yet another adaptive function of LO is no less probable. It should be noted that hydrogen peroxide accumulation is observed during the biofilm growth of bacterial cultures [19]. It is supposed that hydrogen peroxide produced with the involvement of LO catalyzes programmed cell death in the lyzed subpopulation under stress conditions predetermining the biofilm growth of bacteria. It has been shown for representatives of *Marinomonas, Mediterranea, Pseudoalteromonas*, and *Chromobacterium*. The authors believe that hydrogen peroxide induces intrapopulation rearrangements for the intact cells of another subpopulation resulting in emergence of phenotypic variants of the bacteria, which are more resistant to external stressor impacts [19].

The content of amino acids in the growth medium of the fungus *Trichoderma* cf. *aureoviride* Rifai VKM F-4268D during the synthesis of L-lysine-α-oxidase

Growth time, days	Amino acids, mg/L					
	Lysine	Asparagine	Threonine + serine	Glutamine	Glycine	Alanine
0	traces	traces	traces	traces	traces	traces
1	11.9	9.24	8.3	10.15	4.4	5.7
2	7.8	11.8	8.5	11.8	5.3	8.3
3	0	10.73	20.9	13.2	5.15	18.96
4	0	9.64	19.58	18.4	3.5	11.2
5	0	9.56	17.7	23.7	3.1	10.42
6	0	8.59	12.58	26.1	2.5	8.52
7	0	6.2	8.2	27.1	2.1	7.3
9	0	2.7	6.6	29.3	1.8	5.1
10	0	2.8	3.5	33.8	1.68	3.79

Note: The growth in submerged culture on wheat bran in the presence of NaCl.

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