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EXPERIMENTAL ARTICLES

Enzymes of the Xylotrophic Basidiomycete *Lentinus edodes* F-249 in the Course of Morphogenesis

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Abstract—It has been shown that the fungus *Lentinus edodes* grown on a solid wort agar substrate produces intracellular enzymes, including Mn-dependent peroxidase, laccase, and tyrosinase as a family of isoforms. The composition of the complex (containing one to four forms of each enzyme) varied during the basidiomycete life cycle. The activity of oxidases was maximal at the stage of nonpigmented mycelium and at the stages of a brown mycelial mat and a fruit body. The activity of tyrosinase increased in the course of mycelium pigmentation and had two maxima: at the stage of a brown mycelial mat and at the stage of a fruit body. Laccase and tyrosinase activities were shown to increase sharply upon addition of oak sawdust extract to the culture medium as compared with the enzyme activities of mycelium grown on wort agar alone. It was established that the effect of phenol oxidase substrates on the growing mycelium consists in a twofold acceleration of the process of morphogenesis in the fungus *L. edodes*.

Key words: stages of morphogenesis, *Lentinus edodes*, laccase, tyrosinase, Mn-dependent peroxidase.

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Fungal organisms develop in direct contact with the environment; hence, they are permanent objects of physical and chemical stressors, which trigger the processes of differentiation in fungi. Under the influence of external factors in the course of development, anatomical differentiation proceeds in parallel with "chemical differentiation"; i.e., new metabolic pathways are formed in response to stress. Cessation of growth and transition to the stationary phase are known to be coupled with the biosynthesis of secondary metabolites in many fungi. Secondary metabolites are not characteristic of animals but are inherent in microorganisms, plants, and fungi. The biosynthesis of secondary metabolites is one of the many processes accompanying cell differentiation [1]. The molecular mechanisms underlying both processes (differentiation and synthesis of secondary metabolites) are still far from being deciphered, but it is obvious that the occurrence of secondary metabolites is associated with the functioning of enzymes, the synthesis and activation of which are induced by stress factors [2].

Xylotrophic basidiomycetes are a group of higher fungi which occupies a significant place in the structure of plant and forest biocenoses, because their main function is degradation of lignocellulose substrates. These fungi attract attention as participants in the processes of plant waste biodegradation and as producers of enzymes and a unique complex of bioactive substances [3]. Laccases, Mn-dependent peroxidases, and tyrosinases are widespread in xylotrophs; they are involved in the degradation of lignocellulose substrates and in synthesis of pigments and melanins [4–6]. Most basidiomycetes belong to the white rot fungi possessing a lignolytic enzyme complex, the main enzymes of which are Mn-dependent peroxidase and laccase. Being excreted into the environment, these enzymes participate in lignin degradation and in synthesis and degradation of humic substances [7, 8].

Important physiological and ecological functions of laccases, Mn-dependent peroxidases, and tyrosinases necessitate not only the study of participation of these enzymes in the biochemical reactions of degradation of lignocellulose substrates but also elucidation of their role in fungal metabolism and peculiarities of cytodifferentiation.

Lentinus edodes (Berk.) Sing [*Lentinula edodes* (Berk.) Pegler] (shiitake) arouses particular interest not only because of the high nutritive value and taste properties of fruit bodies, but also because this xylotrophic basidiomycete is used for production of a series of valuable medical preparations, which have led to indispensable drugs with no toxic effect on the human organism [9–12]. However, there are no data in either Russian or foreign literature on detection and analysis of the intra-

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cellular enzymes typical of particular morphological structures of this fungus.

The goal of the present work was to study the composition of intracellular phenol oxidases synthesized by the fungus *L. edodes* at the stages of morphogenesis specific for this fungal species, in order to reveal the enzymes characterizing the given morphological structures.

MATERIALS AND METHODS

The fungus and cultivation conditions. Basidiomycete *Lentinus edodes* (Berk.) Sing [*Lentinula edodes* (Berk.) Pegler] (shiitake) F-249 from the collection of higher basidial fungi of the Chair of Mycology and Algology, Moscow State University was used in the work. The fungal culture was maintained on wort agar at 4°C.

Solid-phase cultivation of *L. edodes* was carried out on a medium with agarized beer wort (4° according to Balling) and on wort agar medium with an oak extract [13]. The extract was obtained as follows: dry oak sawdust was submerged in water (100°C), left overnight at room temperature, filtered, and autoclaved for 30 min at 0.5 atm; 10% of the extract was added to the cultivation medium. The cultivation was carried out in petri dishes at the optimal temperature for mycelium growth of this species (26°C) [14].

Nonpigmented mycelium of *L. edodes* (days 7, 14, and 28 of cultivation), pigmented mycelium (day 40), a brown mycelial mat (day 50), primordia (day 60), and fruit bodies were used in the study. The fruit bodies of *L. edodes* were formed on petri dishes with wort agar 60–70 days after inoculation [15].

Detection of oxidase activity during mycelium growth. Qualitative assessment of the oxidase activity in *L. edodes* was carried out on petri dishes in the course of solid-phase cultivation on the medium with agarized brewing beer wort (with and without oak extract) on day 7, 14, and 28. The surface of growing mycelium was covered with 2,2-azine-*bis*(3-ethylbenzothiazoline-6-sulfonate) (ABTS) in 50 mM Na–tartrate buffer (pH 4.5). ABTS oxidation was detected by green staining of the mycelium [16]. Additionally, the presence of oxidase activities was tested using 0.2% dimethoxyphenol (DMOP) [17] and 0.02% syringaldazine [18]. The appearance of brown and bright-pink coloration of the mycelium was observed in the former and latter case, respectively.

Conditions of enzyme isolation. In order to obtain intracellular enzymes, the fungus was grown to a certain stage of morphogenesis, removed from the medium, washed with distilled water, dried at 25°C to a constant weight, weighed, and mechanically ground in a porcelain mortar with quartz sand in order to disrupt the cell envelope; the enzymes were extracted from homogenized dry mycelium (20 mg) with 2 ml of 20 mM Na–K phosphate buffer (pH 6.0) for 2 h; the homogenate was then centrifuged at 12000 *g* for 15 min, and the supernatant was separated from the pellet and filtered.

Intracellular enzymes were obtained from wet fungal mycelium according to a similar procedure; since the mass of mycelium samples decreased 40-fold during drying, wet homogenized mycelium (800 mg) was treated with 2 ml of 20 mM Na–K phosphate buffer (pH 6.0).

Determination of enzyme activity. Enzyme activities were detected at 18°C in a Specord M 40 spectrophotometer (Carl Zeiss, Germany). Laccase activity was determined from the rate of oxidation of 0.2 mM ABTS (Sigma, USA) in 50 mM Na–tartrate buffer (pH 4.5). Oxidation of ABTS to a stable cationic radical was measured by the increase in absorbance at 436 nm (ε_{436} 29300 M^{-1} cm^{-1}) [17]. Mn-dependent peroxidase activity was determined by the rate of oxidation of 0.2 mM ABTS (Sigma, USA) in 50 mM sodium tartrate buffer (pH 4.5) with 0.1 mM H_2O_2 and 0.2 mM $Mn^{(2+)}$ added to the reaction mixture. ABTS oxidation to a stable cationic radical was measured by the increase in absorbance at 436 nm (ε_{436} 29300 M^{-1} cm^{-1}) [19].

Tyrosinase activity was determined by the rate of oxidation of 2 mM L-dihydroxyphenylalanine (L-DOPA, Serva, Germany) in 50 mM Tris–HCl buffer (pH 7.5). L-DOPA oxidation to DOPA quinone was measured by the increase in absorbance at 475 nm (ε_{475} 3700 M^{-1} cm^{-1}) [20].

Reaction time was 5 min in all cases. The amount of enzyme catalyzing transformation of 1 μmol of the substrate or formation of 1 μmol of the product per minute was taken as a unit of activity and expressed as $\mu molmin^{-1}$ mg^{-1} protein.

Protein assay. Protein concentration was determined by the method of Bradford [21].

Electrophoresis in polyacrylamide gel. The composition of the proteins obtained by extraction at each stage of the fungus development was examined by electrophoresis in 12% polyacrylamide gel (PAGE) under nondenaturing conditions (without addition of SDS and β-mercaptoethanol) [22]. The proteins were visualized by staining with Coomassie Blue R-250.

Specific PAGE staining to reveal oxidases was made in the reaction mixtures containing the following: 1% acetic acid (1 ml); 0.2% *ortho*-dianisidine (50 mg); and 50 mM sodium tartrate buffer (pH 4.5) (50 ml). Brown–red staining of the bands corresponding to laccase appeared within 15 min of gel incubation in the reaction mixture [23].

PAGE staining for Mn-dependent peroxidase activity was carried out in the above reaction mixture with additionally introduced 0.1 mM H_2O_2 and 0.2 mM $MnSO_4$. Brown–red staining of the bands corresponding to Mn-dependent peroxidase appeared within 20 min of gel incubation [19].

PAGE staining for tyrosinase activity was made in a reaction mixture of the following composition: 10 mg of L-DOPA and 50 mM of Tris–HCl buffer (pH 7.5) (25 ml). Greenish-brown bands corresponding to tyrosinase appeared within 10 min [20].

RESULTS AND DISCUSSION

Oxidase activity of *L. edodes* growing mycelium. Oxidase activity was detected by the ability to oxidize ABTS, dimethoxyphenol, and syringaldazine on petri dishes with the fungus culture on the medium with agarized brewing beer wort (with and without oak extract) on days 7, 14, and 28 of growth. Positive reaction with ABTS, dimethoxyphenol, and syringaldazine was revealed in all cases. The most intensive staining, which appeared 15 min after the substrate application to the growing culture, was observed in 14-day mycelium cultivated on wort agar with oak extract.

Enzymatic activity of dry mycelium extracts at different stages of morphogenesis. For the analysis of the enzymes during the whole life cycle of the fungus *L. edodes*, the process of morphogenesis was divided into the following stages: nonpigmented mycelium, pigmented mycelium, brown mycelial mat, primordium, and fruit bodies. All these stages are clearly traceable and typical of this basidiomycete.

Laccase, Mn-dependent peroxidase and tyrosinase activities were tested in dried homogenized samples of all the morphological structures of the fungus. Neither laccase nor Mn-dependent peroxidase activities were revealed at any stage of morphogenesis. This may be due to inactivation of the enzymes under the conditions of isolation and storage applied in the present work (drying, freezing, and long-term storage). However, tyrosinase activity was found at all stages of morphogenetic development of the basidiomycete. Its time course was as follows: insignificant tyrosinase activity began to appear on day 14 of cultivation; it increased in the course of culture development. The highest enzymatic activity was observed in the pigmented structures of the fungus, with the maximum at the stage of a brown mycelial mat. The obtained results are in good agreement with the literature, reporting a close relation of tyrosinase to mycelium pigmentation [24, 25]; the brown mycelial mat (the stage preceding fructification) is the most pigmented structure of *L. edodes* morphogenesis.

However, PAGE under nondenaturing conditions and specific gel staining for Mn-dependent peroxidase and tyrosinase activities made it possible to visualize the protein bands corresponding to these enzymes in fungal extracts at all stages of morphogenesis. Mn-dependent peroxidase was found to be produced as a family of isoforms. Three forms of Mn-dependent peroxidase were observed in nonpigmented mycelium, beginning from day 14 of cultivation; two of them were of very close molecular weights. Two forms of Mn-dependent peroxidase, also with close molecular weights and corresponding to those of the previous stage, were found in the pigmented mycelium. At the stage of brown mycelial mat, the Mn-dependent peroxidase forms typical of nonpigmented and pigmented mycelium were absent, but three other forms appeared. Four enzyme forms were revealed at the stage of a primordium; two of them were also present at the stages of nonpigmented and pigmented mycelium as well as at the fruit body stage. In addition, two forms of Mn-dependent peroxidase typical of the brown mycelial mat appeared again at the stage of a fruit body. It should be noted that the finding of several forms of Mn-dependent peroxidase in *L. edodes* was not unexpected. Previous works have reported the presence of families of ligninolytic enzyme isoforms in white rot fungi [26, 27].

Similar to Mn-dependent peroxidase, tyrosinase appears already in a 14-day culture and is present at all stages of the life cycle of the basidiomycete *L. edodes*. Two tyrosinase forms were present both in nonpigmented mycelium on day 28 of cultivation and at the stage of a brown mycelial mat, although their molecular weights were different. The pigmented mycelium, primordium and fruit body each had one tyrosinase form of the same molecular weight.

Laccase was not found under the given experimental conditions.

Enzymatic activity of wet mycelium extracts at different stages of morphogenesis. The picture of enzyme activities obtained with dry mycelium samples was incomplete (probably due to degradation of the enzymes under such conditions of obtaining and storage); therefore, similar studies were carried out with wet samples of mycelium and morphological structures of the fungus *L. edodes*.

In the course of solid-phase cultivation on wort agar, the fungus produces a complex of enzymes including Mn-dependent peroxidase, laccase, and tyrosinase. Figure 1 presents the dynamics of activity of Mn-dependent peroxidase, laccase, and tyrosinase under solid-phase growth conditions. Laccase and Mn-dependent peroxidase activities were revealed in wet culture extracts obtained on days 7 and 14 of cultivation. The maximum activities of these enzymes for nonpigmented mycelium were reached on day 14; no signs of activity were found on day 28. Enzyme activities recovered in the course of mycelium pigmentation. The second peak of Mn-dependent peroxidase activity occurred on day 50 of cultivation; this time corresponds to the formation of a brown mycelial mat. The activity dropped on day 60 (the time of primordia formation) and was absent in fruit bodies. Laccase activity gradually increased from day 40 to day 70 of cultivation and its second maximum occurred on day 70, the time of fruit body formation. An insignificant tyrosinase activity appeared in nonpigmented mycelium by day 14 of cultivation; it became noticeably higher on day 28 and

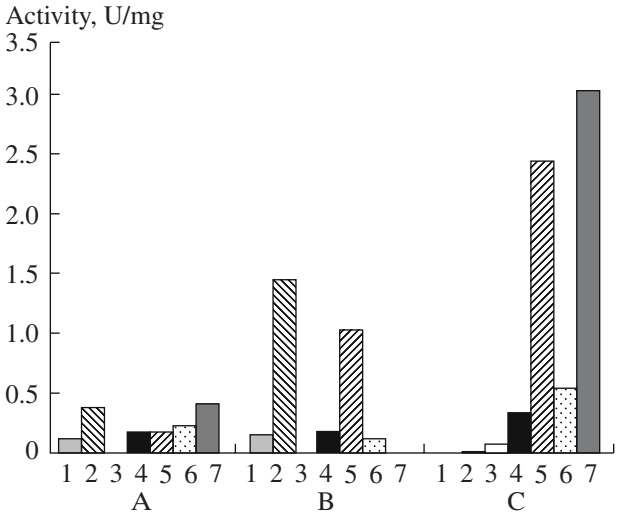

Fig. 1. Dynamics of laccase, Mn-dependent peroxidase, and tyrosinase activities in *L. edodes* solid-phase culture at different stages of morphogenetic development. 1, non-pigmented mycelium (7-day culture); 2, non-pigmented mycelium (14-day culture); 3, non-pigmented mycelium (28-day culture); 4, pigmented mycelium; 5, brown mycelial mat; 6, primordium; 7, fruit bodies. A, laccases; B, Mn-dependent peroxidases; C, tyrosinases.

increased in the course of pigmentation with two maxima: at the stage of a brown mycelial mat and at the stage of a fruit body. The obtained data on the dynamics of tyrosinase activity correlate with the results of the previous experiment; however, tyrosinase activity in wet culture extracts was four times higher in the extracts of the brown mycelial mat and about 100 times higher in fruit body extracts.

The composition of the oxidase complex also changed depending on the time of cultivation. Figure 2 shows the results of electrophoresis in nondenaturing conditions. On day 7 of cultivation, the complex contained two forms of laccase and two forms of Mn-dependent peroxidase. On day 14–28, there were four forms of laccase and three forms of Mn-dependent peroxidase; however, on 28 of cultivation no activity of these enzymes was revealed. In pigmented mycelium extracts, four laccases and two Mn-dependent peroxidases were revealed; three laccases and three Mn-dependent peroxidases were present at the stage of a brown mycelial mat; three laccases and four Mn-dependent peroxidases occurred in the primordium; a single laccase (the form dominating in the complex independent of the time of cultivation) and four forms of Mn-dependent peroxidase were revealed in fruit body extracts. Usually, laccases are produced as several isoenzymes (this phenomenon is known for other lignolytic enzymes). Laccases from various fungi are different both in the number of enzyme forms and in the physicochemical properties of individual isoforms [28, 29].

The above data on the Mn-dependent peroxidase complex are in agreement with the results of the previous experiment. In addition, similar results have been obtained for the composition of the tyrosinase complex.

Dependence of the oxidase activity of the fungus on the presence of natural substrates in the growth medium. Under natural conditions, the xylotrophic basidiomycete *L. edodes* grows and develops on substrates rich in lignin; hence, the activity of some enzymes was studied under solid-phase cultivation on a medium containing a sawdust extract. Oak, poplar, elm, and maple extracts were taken for comparison.

It has been shown that laccase and tyrosinase activities increased sharply upon addition of oak sawdust extract to the culture medium, as compared with the enzymatic activity of mycelium grown on wort agar without the extract. Poplar, elm, and maple wood pulp extracts had no such effect (data not presented). The activity observed at cultivation on the above substrates, although slightly higher than at cultivation on wort agar alone, was more than 10 times less than the maximum reached at cultivation in the medium with an extract from oak sawdust (which is the natural substrate most often colonized by shiitake in vivo). Besides, laccase

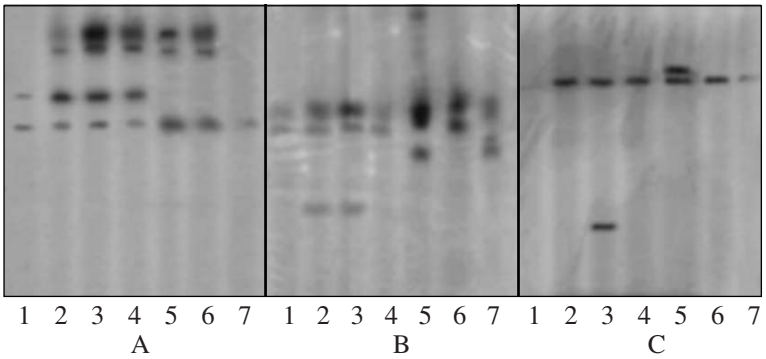

Fig. 2. Electrophoresis under nondenaturing conditions of *L. edodes* oxidases obtained by solid-phase cultivation at different stages of morphogenetic development. Designations are as in Fig. 1.

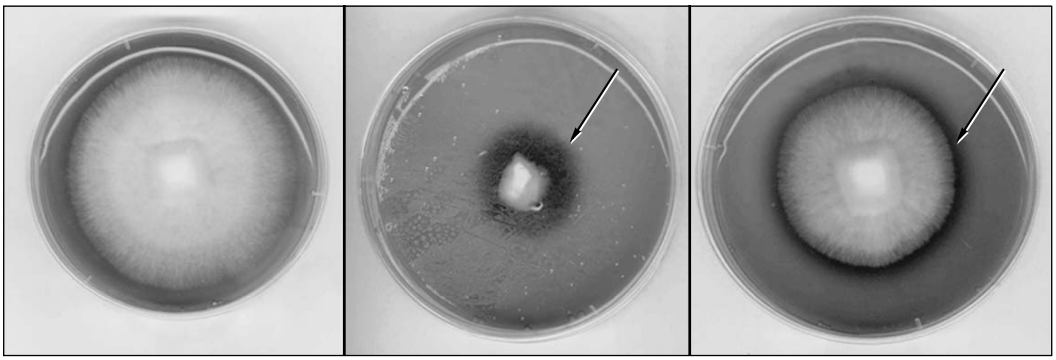

Fig. 3. Effect of the presence of a natural substrate in the medium on oxidase activities at *L. edodes* solid-phase cultivation. A, mycelium on wort agar medium (14-day); B, mycelium on wort agar medium with the addition of an oak extract (7-day); C, mycelium on wort agar medium with the addition of an oak extract (14-day); the arrow marks the products of lignin degradation by *L. edodes* oxidases.

and tyrosinase activities were already observed on day 7 and reached their maximum on day 14 of cultivation.

The activity of Mn-dependent peroxidase on day 7 of cultivation with and without oak sawdust extract was comparable. However, on day 14 the activity was completely absent and appeared again on day 28; it was two times higher than in the 7-day mycelium.

It is known that laccase is required for the initial attack on the polymer molecule of lignin [4]. Addition of oak extract, i.e., introduction of natural lignin into the culture medium, induces a response reaction of the fungus: laccase activity aimed at substrate degradation increases by several orders of magnitude. This is probably the case because the dark ring around the growing mycelium, which can be seen on petri dishes, may consist of lignin degradation products (Fig. 3). Apparently, the basidiomycete culture does not need large amounts of laccase on the media without lignin. Previous studies on the medium without lignin have shown much lower laccase activity, supporting this assumption.

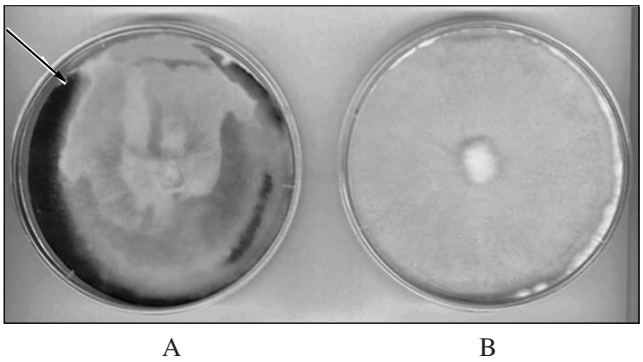

Fig. 4. The effect of ABTS on formation of a brown mycelial mat in *L. edodes* solid-phase culture. A, mycelium with ABTS (28 days of cultivation); B, mycelium without ABTS (28 days of cultivation); the arrow marks the brown mycelial mat.

Participation of phenol oxidases in morphogenesis. The appearance of a brown mycelial mat precedes fructification, which is typical of the given culture. Under usual conditions (without any phenol substrate), this structure, which is an interlacing of thick pigmented hyphae, is formed on the 50th day of cultivation. An interesting peculiarity was observed upon introduction of ABTS (Fig. 4), dimethoxyphenol, and syringaldazine into the culture medium: the formation of a brown mycelial mat was observed on day 20 after the growing 7-day mycelium was exposed to their action; i.e., morphogenesis of *L. edodes* proceeded twice faster. Apparently, phenol oxidase substrates activated the action of laccase, resulting in mediated or direct involvement of the mechanisms of formation of a brown mycelial mat.

These findings are in good agreement with the literature data pointing out the connection between laccase formation in some fungi, including *L. edodes*, and the synthesis of melanins and pigment formation in the structures, which are more dense than a simple mycelial aggregate [4]. In this case, it is the brown mycelial mat, a dense structure rich in pigments, which seems to perform a protective function in the beginning of fruit body formation under unfavorable environmental conditions. Pigments serve as a kind of "screen" from excessive illumination and reduce the permeability of cell walls for toxic agents and pathogens. Fruit body formation is often accompanied by the activity of phenol oxidases. This process, in its turn, is closely related to the formation of extracellular pigments and proceeds in parallel with oxidative polymerization of cell envelope components, strengthening the intercellular adhesion [30].

Thus, the above data makes it possible to conclude that the fungus produces a complex of intracellular enzymes, including Mn-dependent peroxidase, laccase,

and tyrosinase, under solid-phase cultivation on wort agar. The maximum activity of oxidases has two peaks: the first peak occurs on day 14 of cultivation; the second peak of Mn-dependent peroxidase activity corresponds to the time of formation of a brown mycelial mat, and the second maximum of laccase activity falls on the time of a fruit body formation. Tyrosinase activity increases in the course of mycelium pigmentation and also has two maxima: at the stage of a brown mycelial mat and at the stage of a fruit body. In the cultures, the composition of the oxidase complex changes with time. Laccases and Mn-dependent peroxidases are produced as a family of isoforms; the complex comprises two to four forms of each enzyme in different morphological structures of *L. edodes*. Only at the stages of nonpigmented mycelium and a brown mycelial mat, tyrosinase has two forms; one form exists at other stages of morphogenesis.

It has been revealed that the addition of oak sawdust extract to the culture medium results in a sharp increase in laccase and tyrosinase activities as compared with the enzyme activity of mycelium grown on wort agar without the extract. This fact confirms the assumption of their involvement in lignin biodegradation and in removal of some secondary metabolites and low-molecular phenols through their conversion into high-molecular nontoxic compounds. The action of phenol oxidase substrates on the growing mycelium was found to accelerate the process of morphogenesis in *L. edodes* at least twofold, which seems to result from the activation of laccase action.

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