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EXPERIMENTAL ARTICLES

Chrysene Bioconversion by the White Rot Fungus *Pleurotus ostreatus* D1

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Abstract—The effect of cultivation conditions on chrysene bioconversion by the fungus *Pleurotus ostreatus* D1 was shown. Under the laccase production conditions, transformation of this polycyclic aromatic hydrocarbon occurs with accumulation of the quinone metabolite. Under both the laccase and versatile peroxidase production conditions, chrysene degradation occurs, with the stages leading to phthalic acid formation and its further utilization. The formation of phthalic acid as a metabolite of chrysene degradation by white rot fungi was revealed for the first time. The data obtained suggest that the laccase revealed on the mycelial surface and the extracellular laccase are probably involved at the initial stages of chrysene metabolism, whereas versatile peroxidase seems to be required for oxidizing the metabolites formed.

Key words: chrysene, *Pleurotus ostreatus* D1, laccase, versatile peroxidase.

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Polycyclic aromatic hydrocarbons (PAHs) are widespread pollutants formed as a result of industrial and natural processes [1]. They are the constituents of coal tar, creosote, and crude mineral oils and are formed during combustion of organic matter [2]. The solubility of PAHs decreases with an increase in their molecular weight, which determines their capacity for accumulation in biological membranes and tissues. PAHs are also bound to soil and sediments and accumulate in the environment [1].

The white rot fungi, the most active destructors of lignin in nature, are also capable of metabolizing a broad spectrum of aromatic xenobiotics, including PAHs [3]. This capacity is linked to their extracellular enzyme system, which includes lignin peroxidase, manganese peroxidase, versatile peroxidase, and laccase [4].

At present, few reports on four-ring PAHs degradation by white rot fungi are available in the literature. The degradation of highly toxic chrysene, with solubility not exceeding 0.002 mg/l, is the least studied area [5]. The main studies are conducted with the fungi cultivated in soil. Thus, for example, it was shown that from 28 to 45% of this substrate was metabolized by different species of the white rot fungi, including *Phanerochaete chrysosporium, Bjerkandera adusta, Irpex lacteus*, and *Pleurotus eryngii* [6]. Moreover, it was noted that individual strains of the same fungal species, *Pleurotus ostreatus*, used from 29 to 42% of chrysene over 15 weeks of cultivation at a starting concentration of 2.8 mg/g of soil [7] and up to 79% of chrysene in a mixture with other PAHs [8].

We did not find any evidence of either mineralization of this substrate or identification of its degradation products in the literature available. Chrysene degradation in submerged cultures, which are often used for metabolite isolation and investigation of regulatory mechanisms, has not been studied. Almost no data exist concerning the production of ligninolytic enzymes in the presence of chrysene.

Investigation of the mechanisms of chrysene degradation by white rot fungi is therefore of great interest. The fungus *Pleurotus ostreatus*, which is known not only as a chrysene degrader [7, 8], but also as one of the most powerful producers of ligninolytic enzymes [9, 10], was selected as the study subject. According to the results of our preliminary investigations, the strain of the fungus *P. ostreatus* D1 metabolized a number of PAHs containing from three to five aromatic rings and was an active laccase producer as well [11].

The aim of this work was to investigate chrysene degradation by the fungus *P. ostreatus* D1 during submerged cultivation.

MATERIALS AND METHODS

The fungus *P. ostreatus* D1 was obtained from the Laboratory of Microbiology and Mycology, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences [12].

Cultivation was carried out in Kirk medium [13] and in a basidiomycetes rich medium for [14]. Chry-

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sene was added on the third day to a final concentration of 50 mg/l as a chloroform solution (100 μl of solution/100 ml of medium). Chloroform (100 μl/100 ml of medium) was added to the control variants. After certain time intervals, the mycelium and the cultivation medium were separated by filtration. The mycelium increment (mg of dry biomass) was determined by weighing and expressed as a percentage of the chrysene-free control variant. The emulsifying activity (E_{48}) of the cultivation medium was determined according to Cooper's method [15].

In order to study chrysene bioconversion under nongrowth conditions, the fungus was cultivated in basidiomycetes rich medium for maximal production of laccase or peroxidase (on days 2–3 and day 20, respectively); the mycelium and the medium were then separated by filtration.

The cultivation medium was then used as a source of extracellular enzymes. Experiments with a crude laccase preparation (10 U/ml) were carried out in 50 mM phosphate buffer (pH 6.0) containing 1% of acetonitrile and 20 μM of chrysene. The experiments with the crude preparation of versatile peroxidase (10 U/ml) were carried out in the same buffer containing 1% of acetonitrile, 20 μM of chrysene with or without 100 μM of $MnSO_4$ and 200 μM of H_2O_2. The reaction mixtures were incubated for 10 days at 29°C. The control samples were prepared in a similar way, with the enzymes inactivated by boiling for 10 min before the addition of chrysene.

The mycelium was washed with 50 mM phosphate buffer (pH 6.0) to remove the extracellular enzyme activity and incubated in the same buffer with 20 μM chrysene for 10 days at 29°C. The control samples were prepared in a similar way, with the mycelium inactivated by 20-min boiling prior to addition of chrysene.

Chrysene and degradation products thereof were extracted from the cultivation medium or reaction mixtures three times with an equal volume of ethyl acetate. The extracts were pooled and evaporated to the minimum volume. The chrysene decrease was analyzed by HPLC (Spectra Series P200, Spectra-Physics Analytical, Inc.) on a Spherisorb S5 PAH column with a Spectra Series UV 100 detector (Thermo Separation Products, United States).

The chrysene bioconversion products were analyzed by thin-layer chromatography (TLC) on the Silufol UV-254 plates (Kavalier, Czech Republic) in a benzene : ethanol (9 : 1) system and by gas chromatography (Shimadzu 2010) on an Equity-1 column (Supelco, United States) with a flame photometric detector. Prior gas chromatographic analysis, the metabolites were methylated with CH_3COCl [16].

The activity of extracellular laccase was tested by the ABTS (diammonium 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonate) oxidation reaction at 436 nm [17]. In order to reveal the activity of laccase on the mycelial surface, 1 g of washed mycelium was placed into an ABTS-containing reaction mixture, from which aliquots were sampled after certain time intervals, and the absorption was measured at 436 nm. The versatile peroxidase activity was tested by the 2,6-dimethoxyphenol oxidation reaction and calculated as the difference between the reactions with and without H_2O_2 [18].

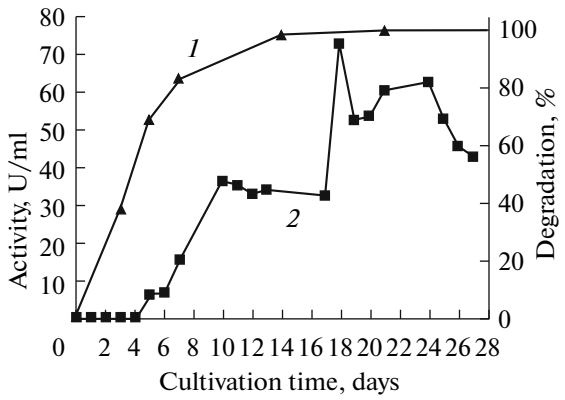

Fig. 1. Chrysene degradation (*1*) and laccase production (*2*) in Kirk medium.

The amount of the enzyme catalyzing the formation of 1 μmol of the product per minute was taken as the activity unit and expressed in conventional units—μmol/(min ml of the enzyme preparation (U/ml) or U/g of mycelium crude weight.

RESULTS AND DISCUSSION

It was revealed that, irrespective of the conditions, the fungus actively metabolized chrysene and no more than 5% of the initial amount was determined in the cultivation medium by the seventh day of cultivation (Figs. 1, 2). Sorption on the mycelium was minimal and did not exceed 5%, which agrees with the data obtained for phenanthrene degradation [14, 19].

When cultivation was carried out in Kirk medium, mycelium growth was inhibited during the first 3–5 days, probably due to the formation of metabolites that were toxic to the fungus. All these compounds were then degraded, and the amount of mycelium in the control and experimental variants leveled off by day 21. Chrysene utilization was accompanied by a yellow coloration of the cultivation medium, which was retained throughout the experiment and disappeared upon addition of a reducer (dithionite), suggesting the presence of quinone intermediates [20]. Similar data on the degradation of another four-ring PAH, pyrene, were found in the available literature. Moen and Hamme showed that, in this case, quinone metabolites also accumulated, with the yellow color disappearing on addition of dithionite [20].

TLC of the cultivation medium extracts demonstrated the presence of a single metabolite with $R_f =$

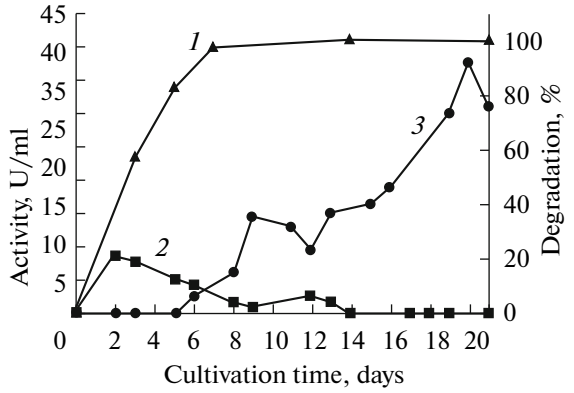

Fig. 2. Chrysene degradation (*1*) and extracellular enzyme production in a rich medium for basidiomycetes: laccase (*2*) and versatile peroxidase (*3*).

0.88 (the R_f of chrysene is 0.93) that was present until the end of the experiment. HPLC showed the disappearance of the peak with a retention time of 21.1 min corresponding to chrysene and the appearance of the metabolite peak with a retention time of 18.7 min. This metabolite was isolated using preparative TLC. Its absorption spectrum showed a shift of the characteristic chrysene maxima at 262 and 270 nm to the long-wave region. The identification of metabolite I will be the subject of further investigations. Under these conditions, we revealed production of one ligninolytic enzyme, laccase, throughout the time of the experiment (Fig. 1).

When *P. ostreatus* D1 was cultivated in a basidiomycetes rich medium, chrysene and/or its degradation products were not toxic to the fungus. The biomass quantity in the experimental and control variants was approximately the same over 3 weeks. We revealed three metabolites with R_f = 0.88 (I), 0.54 (II), and 0.21 (III). By R_f, retention time, and absorption spectrum, metabolite I corresponded to the metabolite revealed during cultivation of the fungus in Kirk medium. However, unlike the experiment in Kirk medium, it was present only up to 14 days, at which point two more metabolites (II and III) were revealed in the cultivation medium. The dark violet luminescence and R_f = 0.21 of metabolite III corresponded to the similar properties of the standard, phthalic acid. This compound was isolated with preparative TLC; its absorption spectrum with a maximum at 280 nm corresponded to that of phthalic acid, and the retention time (8.5 min) determined by gas chromatography corresponded to the retention time of this compound. Based on these data, this metabolite was identified as phthalic acid.

In contrast to cultivation on Kirk medium, in basidiomycetes rich medium, the loss of chrysene was accompanied by the production of two ligninolytic enzymes, namely, laccase over the first 24 h of cultivation and versatile peroxidase at the later stages, when there was practically no initial substance revealed in the cultivation medium (Fig. 2).

In the course of chrysene degradation in basidiomycetes rich medium, foam formation occurred, this probably indicating biosurfactant production. Similar results have been described only in one publication for the fungus *Phanerochaete chrysosporium* cultivated in the presence of pyrene [21]. These authors suggested that foam formation was linked to the formation of a biosurfactant, which contributes to pyrene dissolution in the aqueous medium [21]. We checked the emulsifying activity of the medium after cultivation of the fungus *P. ostreatus* D1. In the control variant (without PAH), it was about 1%, whereas in the presence of chrysene it reached 48.4%. Moreover, it was noted that peroxidase production in the presence of chrysene was simultaneous with an increase in the emulsifying activity of the medium. This may indicate that the emulsifying agent produced by the fungus is required not only for chrysene dissolution, but also for the catalytic activity of versatile peroxidase, as occurs in the case of phenanthrene oxidation by manganese peroxidase in the presence of Tween-80 [20].

Studies of phthalic acid degradation under different conditions showed that this compound could be metabolized by the fungus *P. ostreatus* D1, irrespective of the cultivation conditions. According to gas chromatography data, the loss of phthalic acid was 65.6 ± 1.8 and 92.4 ± 0.11% over 14 days of cultivation in Kirk medium and basidiomycetes rich medium, respectively. Analysis of the literature data [22] permitted the speculation that the phthalic acid formed as a result of chrysene degradation may be involved in the basal metabolism.

The involvement of different groups of enzymes in PAHs degradation by white rot fungi is presently widely discussed. In our opinion, an important pool of enzymes is not taken into account, namely, the laccases revealed on the mycelial surface. The information about these laccases and their possible functions is still very limited [23]. We suggested that the laccase revealed on the mycelial surface might be involved in the initial attack of a PAH molecules, because it takes some time for extracellular laccase to be produced into the cultivation medium. To test this suggestion, we studied the catalytic activity of *P. ostreatus* D1 intact mycelium. Mycelium obtained upon cultivation on a PAH-free basidiomycetes rich medium possessed 1.24 U of laccase/g of crude mycelium weight. Cultivation on the same medium in the presence of chrysene increased the laccase activity on the mycelial surface 3.4-fold. When the washed mycelium was incubated with chrysene, the concentration of this PAH was found to decrease by 21.7 ± 2.8%. HPLC revealed the presence of a reaction product peak with a retention time of 18.7 min, which was similar to that we revealed under the growth conditions.

In addition, chrysene bioconversion by crude enzyme preparations was studied. On incubation with

a crude laccase preparation, the decrease in chrysene concentration was 62.7 ± 2.7%. HPLC also enabled us to reveal the presence of the reaction product peak with retention time of 18.7 min. The crude versatile peroxidase preparation decreased the chrysene concentration by 12.6 ± 1.5% in the presence of H_2O_2. Addition of $MnSO_4$ to the reaction mixture decreased the chrysene concentration by 24.2 ± 0.7%. The study of phthalic acid oxidation by crude preparations of these enzymes showed that 60 ± 6.7% of this substrate was oxidized by versatile peroxidase without $MnSO_4$ in the reaction mixture. Addition of $MnSO_4$ did not lead to an appreciable increase of oxidation. The laccase crude enzyme preparation did not oxidize phthalic acid.

Thus, we showed that chrysene bioconversion by the fungus *P. ostreatus* D1 depends on the cultivation conditions. Under conditions in which only laccase is produced, transformation of this PAH probably occurs, as evidenced by accumulation of the quinone metabolite I. Under conditions in which both laccase and versatile peroxidase are produced, degradation occurs, which includes the stages leading to phthalic acid formation and further utilization thereof. It should be emphasized that it is the first time that phthalic acid formation as a metabolite of chrysene degradation by white rot fungi was shown and the emulsifying activity of the biosurfactant formed was determined. It was found that the laccase revealed on the mycelium surface, extracellular laccase, and versatile peroxidase were capable of oxidizing chrysene. Along with this, phthalic acid, a metabolite of chrysene degradation, was oxidized only by versatile peroxidase, whereas this substrate was not available to laccase. The data obtained allow us to suggest that the laccase revealed on the mycelial surface and extracellular laccase may be involved in the initial stages of chrysene metabolism, whereas the versatile peroxidase was probably required for oxidizing the metabolites formed.

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