Ligninolytic basidiomycetes as promising organisms for the mycoremediation of PAH-contaminated Environments

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Abstract. Primary screening of ligninolytic fungi belonging to wood- and soil-inhabiting basidiomycetes revealed their ability to degrade three-ringed PAHs with formation of quinone metabolites at the first stage. The degradative activity was both species and strain specific, and some differences in the “chances” for the formed quinones were found. They were the main end metabolites in the degradation of PAHs by Stropharia rugosoannulata and Agaricus bisporus. During PAH degradation by strains of Trametes versicolor, Pleurotus ostreatus, Schizophyllum commune, and Bjerkandera adusta similar metabolites were detected during the cultivation, but they were utilized further. The results supported the hypothesis that the degree of PAH degradation may depend on the composition of the extracellular ligninolytic complex of the fungi: in the presence of a single ligninolytic enzyme, laccase, the accumulation of quinone metabolites takes place; their further utilization is possible with the participation of ligninolytic peroxidases. The data obtained showed the necessity not only to identify the metabolites formed, but also to study the activity of the basic ligninolytic enzymes. It is important for the correct selection of fungal strains for mycoremediation.

1. Introduction
Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds containing two and more fused aromatic rings. The widespread occurrence of PAHs is due to their generation from the incomplete combustion of pyrolysis of numerous organic materials, such as coal, oil, petroleum, and wood. PAHs exhibit the most structural variety in nature, as compared to other classes of non-halogenated molecules in the biosphere [1, 2]. The fate of PAHs in the environment includes volatilization, photooxidation, chemical oxidation adsorption on soil particles, and leaching [3]. They are difficult to degrade in natural matrices, and their persistence increases with their molecular weight. Because of their toxic, mutagenic, and carcinogenic properties, the US EPA lists 16 PAHs as priority pollutants [1-3].

Many reviews exist on the biodegradation of this class of pollutant. PAHs are degraded by bacteria, fungi, and algae [1-6]. Among the ways to address environmental contamination, biotechnological methods of remediation have serious advantages over physical and chemical methods owing to their ecological feasibility and relatively low cost. In the past decade, mycoremediation, a biotechnology that uses the degradative potential of fungi of various ecological-physiological and taxonomic affiliations, was developed [7, 8]. The main role in this technology belongs to a thorough study of fungal strains being able to effectively degrade pollutants.

Ligninolytic fungi are a physiological rather than a systematic group of fungi which are the most active degraders of lignin in nature. They have certain advantages over other microorganisms,
including high growth rate and high activity of their extracellular enzymes, which catalyze the degradation of lignin [9]. *Phanerochaete chrysosphorium*, *Pleurotus ostreatus*, *Bjerkandera adusta*, and *Trametes versicolor* are most commonly used for the degradation of such compounds owing to their production of ligninolytic enzymes such as lignin peroxidase, Mn-peroxidase, and laccase. The rate of biodegradation depends on culture conditions such as temperature, oxygen, pH, accessibility of nutrients, ionization potentials and concentrations of PAHs, and agitated or shallow culture [1, 10, 11].

Pollutants containing aromatic structures, such as PAHs, can be involved in the oxidation of lignin components of wood. This phenomenon, with species and/or strain specificity, is the reason to search for and study active strains of ligninolytic fungi which can be used for the development of ecological biotechnologies.

2. Material and methods

The main objects of study were 10 strains of *wood-inhabiting (Bjerkandera adusta* MUT 3398, *Schizophyllum commune* IBPPM541, *Pleurotus ostreatus* f. Florida IBPPM540, *Trametes versicolor* DSM11269, *Trametes versicolor* MUT3403, *Pleurotus ostreatus* 336, *Pleurotus ostreatus* MUT2977, and *Pleurotus ostreatus* D1 (Jacquin) P. Kummer) and *soil-inhabiting basidiomycetes* (*Stropharia rugosoannulata* DSM11372 and *Agaricus bisporus* F-8).

The fungi were grown at 24-26°C in a basidiomycetes rich medium [12] with our modifications (g L<sup>-1</sup>): NH<sub>4</sub>NO<sub>3</sub>, 0.724; KH<sub>2</sub>PO<sub>4</sub>, 1.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.0; KCl, 0.5; yeast extract, 0.5; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.001; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.0028; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.033; D-glucose, 10.0; peptone, 10.0. PAHs (anthracene, phenanthrene, or fluorene) were added on day 2 of growth as a chloroform stock solution to a final concentration of 0.05 mg·L<sup>-1</sup>. The control treatments contained 100 μl of chloroform. After 14 days of cultivation, residual PAHs were extracted from the flasks (without separation of the cultivation medium from mycelia) with chloroform (5 ml, three times). The resulting extracts were evaporated and were analyzed as described below. The mycelium was separated from the cultivation medium by filtration. The mycelium increment (mg of dry biomass) was determined by weighing.

The PAHs and their metabolites were analyzed by GC (Shimadzu 2010) with a flame photometric detector. Compounds were separated by using an HP5 column (Agilent) and helium as the carrier gas. The column temperature was kept at 200°C for 3 min, then programmed to increase to 270°C at a rate of 15°C min<sup>-1</sup>, and finally kept at 270°C for 2 min. Before GC analysis, 2,2′-diphenic and phthalic acids were methylated with CH<sub>3</sub>COCl. Commercial anthracene (RT=4.15 min), 9,10-anthaquinone (RT=5.39 min), phenanthrene (RT=4.08 min), 9-phenanthrol (RT=6.33 min), 9,10-phenanthrenequinone (RT=6.86 min), 2,2′-diphenic acid (RT=6.7 min), and phthalic acid (RT=8.99 min) were used as the marker compounds to identify the residual PAHs and metabolites. GC analysis was carried out at the Center for the Collective Use of Research Equipment in the Field of Physicochemical Biology and Nanobiotechnology, “Simbioz” (Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences).

Laccase activity was measured by the oxidation rate for 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) at 436 nm (ε=29300 M<sup>-1</sup>·cm<sup>-1</sup>), according to Niku-Paavola et al. [13]. Mn-peroxidase was measured by the oxidation rate for 2,6-dimethoxyphenol with H<sub>2</sub>O<sub>2</sub> and Mn<sup>2+</sup> at 468 nm (ε=14800 M<sup>-1</sup>·cm<sup>-1</sup>), according to Heinfling et al. [14]. Peroxidase activity was calculated as the difference between the values for 2,6-dimethoxyphenol oxidation with and without H<sub>2</sub>O<sub>2</sub>. Lignin peroxidase was measured by the formation of the oxidation product of veratryl alcohol at 310 nm (ε=9300 M<sup>-1</sup>·cm<sup>-1</sup>) [15]. One unit of enzyme activity (U·ml<sup>-1</sup>) is defined as the amount of enzyme that oxidizes 1 μM of substrate per min.

All experiments were run in triplicate; each has been repeated at least three times. Standard statistical methods were used to determine the mean values and standard deviations (SD). Data were processed with Microsoft Excel 2003.
3. Results and discussion
We studied 10 strains of ligninolytic fungi belonging to the genera Agaricus, Bjerkandera, Pleurotus, Schizophyllum, Stropharia, and Trametes. All the basidiomycetes actively degraded PAHs, as evidenced by decreases in their concentrations in the cultivation medium (Figure 1) and by the formation of the corresponding metabolites. The sorption of PAHs by the fungal mycelium was insignificant and did not exceed 5-7%. From the three tested compounds, anthracene was the least available to all fungi. The degradation of this compound varied from 39.8 to 100% for Sch. commune and P. ostreatus, respectively. The Pleurotus and Trametes strains metabolized them more intensely (up to 90%). Phenanthrene and fluorene were degraded more actively by all studied fungi. The decrease in their concentration with P. ostreatus D1 was about 100%.

According to their availability to the fungal cells, the PAHs were ranked in the following order: anthracene → phenanthrene → fluorene. Anthracene, with the lowest ionization potential (7.43 eV), has a low solubility (0.07 mg L⁻¹). Perhaps this makes the molecules of anthracene poorly available for the fungal attack. The well-soluble phenanthrene (1.8 mg L⁻¹) has a sufficiently high ionization potential (8.03 eV) and, as a consequence, is well accessible to the fungi. Fluorene has a "successful" combination of a low ionization potential (7.88 eV) and high solubility (1.98 mg L⁻¹), which makes it the best accessible PAH among these studied. Comparison of the obtained data with the physicochemical properties of these substances suggests that in this case, a coupling of two factors – solubility and ionization potentials – is essential.

The degradative activity was both species and strain specific. For example, among the Pleurotus strains tested, P. ostreatus D1 was more active, whereas T. versicolor MUT was more active than T. versicolor DSM.

SD values do not exceed 5%

**Figure 1.** PAH elimination by the fungi studied: ANT – anthracene, PHE – phenanthrene, FLU – fluorene

It is well-known that the first and main metabolites of PAHs are the corresponding quinones [11, 16-23]. Two possible “chances" for the formed quinones are known. First, they can be the end metabolites of PAH degradation; their accumulation was found in Phanerochaete chrysosporium [16, 20], B. adusta [17, 20], and Pycnoporus sanguineus [18]. The second possibility is the subsequent
utilization of quinone metabolites, as shown in *P. ostreatus* [17], *P. eringii* [19], *Ph. laevis* [21, 22], and *Polyporus* sp. S133 [23-24]. In this case, phthalic and benzoic acids were metabolites from “deep” degradation of fluorene [19] and anthracene [24]. The mechanism of the degradation of phenanthrene by *Polyporus* sp. S133 was determined through identification of several metabolites; 9,10-phenanthrenequinone, 2,2′-diphenic acid, salicylic acid, and catechol. 9,10-Oxidation and ring cleavage to give 9,10-phenanthrenequinone is the major fate of phenanthrene. The identification of 2,2′-diphenic acid in culture extracts indicates that phenanthrene was initially attacked through dioxygenation at C₉ and C₁₀ to give cis-9,10-dihydrodiol. Dehydrogenation of phenanthrene-cis-9,10-dihydrodiol to produce the corresponding diol, followed by ortho-cleavage of the oxygenated ring, produced 2,2′-diphenic acid [23].

In our experiments, some differences were found also. The main end metabolites in the degradation of PAHs by *Str. rugosoannulata* and *A. bisporus* were their quinone derivatives; 9,10-anthraquinone, phenanthrene-9,10-quinone, and 9-fluorenone. During PAH degradation by both strains of *Trametes*, the traces of the corresponding quinones were found. With the *P. ostreatus* strains, *Sch. commune*, and *B. adusta*, similar metabolites were detected during the cultivation, but they disappeared when the cultivation time was extended. This indicated that they were utilized further. Indeed, GC-analysis revealed phthalic acid as the metabolite resulting from “deep” degradation of anthracene and fluorene by *P. ostreatus* strains. 2,2′-Diphenic and phthalic acids were produced by “deep” phenanthrene degradation by these fungi. These metabolites can be included in the basal metabolism of the fungi.

It is known that the ligninolytic enzyme system catalyzes the key stages of PAH degradation by fungi. The addition to the cultivation medium of some compounds can simultaneously enhance the degradation of PAHs and the activities of ligninolytic enzymes [10]. Examining of the activity of the main enzymes of the ligninolytic complex in the culture fungi’s medium revealed the production of laccase, Mn-peroxidase, and lignin peroxidase. Differences in the activity and composition of the ligninolytic complexes of individual species were noted: *P. ostreatus* strains, *T. versicolor* strains, and *Sch. commune* produced laccase and Mn-peroxidase; *Str. rugosoannulata* and *A. bisporus* produced only laccase under the experimental conditions, and *B. adusta* produced lignin peroxidase and Mn-peroxidase (table 1).

**Table 1.** Activity of the fungal enzymes.

| Fungus                | Laccase Activity, U·ml⁻¹ | Mn-peroxidase Activity, U·ml⁻¹ |
|-----------------------|--------------------------|-------------------------------|
|                       | control | ANTa | PHE | FLU | control | ANTa | PHE | FLU |
| *A. bisporus*         | 54.2    | 71.2  | 38.3 | 56.4 | –        | 3.6   | 3.2  | 3.4  | 21.5 |
| *B. adusta*           | –       | –     | –    | –    | 3.6      | 3.2   | 3.4  | 21.5 |
| *P. ostreatus* f. Florida | 49.9    | 7.3   | 46.2 | 16.9 | 242.5    | 38.4  | 78.8 | 14.2 |
| *P. ostreatus* MUT   | 108.4   | 24.9  | 174.7 | 158.2 | 392.6    | 118.5 | 494.9 | 292.0 |
| *P. ostreatus* 336   | 98.9    | 33.2  | 29.8 | 5.6  | 145.5    | 25.9  | 7.1  | 12.2 |
| *P. ostreatus* D1    | 9.2     | 8.0   | 24.0 | 0.3  | 6.0      | 75.0  | 21.0 | 6.7  |
| *Sch. commune*       | 26.8    | 11.1  | 13.2 | 15.9 | 86.3     | 3.0   | 27.5 | 3.2  |
| *Str. rugosoannulata*| 2.1     | 5.1   | 4.8  | 2.5  | –        | –     | –    | –    |
| *T. versicolor* DSM  | 134.1   | 125.9 | 141.6 | 73.7 | 24.1     | 9.6   | 15.3 | 10.4 |
| *T. versicolor* MUT  | 167.4   | 103.4 | 176.3 | 164.1 | 179.8    | 128.5 | 64.1 | 86.4 |

*ANT* – anthracene, *PHE* – phenanthrene, *FLU* – fluorene

No correlation between enzyme activities and percentage of PAH degradation was found in all fungi studied. On the whole, the activity of Mn-peroxidase in the *P. ostreatus* strains was higher than that of laccase. But in the *T. versicolor* strains, laccase activity was higher than that of Mn-peroxidase. In any case, the presence of the PAHs and/or their degradation products decreased the activities of the ligninolytic enzymes (table 1). The activity of lignin peroxidase of *B. adusta* was unaffected by the
PAHs or their metabolites in the cultivation medium: 36.8 U·ml\(^{-1}\) (no PAHs), 42.3 U·ml\(^{-1}\) (with anthracene), 39.5 U·ml\(^{-1}\) (with phenanthrene), and 37.2 U·ml\(^{-1}\) (with fluorene).

It is known that PAHs are available for oxidation by all three ligninolytic enzymes; the corresponding quinones are the products of these reactions [25-27]. Correlation of our data on the metabolites formed during PAH degradation with those on the production of enzymes allows us to conclude that, ligninolytic enzymes catalyze the initial attack on the molecules of these pollutants. However, in the presence of a single ligninolytic enzyme, laccase, their accumulation takes place, and further utilization is possible with the participation of Mn-peroxidases. In the case of B. adusta the first attack on the molecules of PAHs can be catalyzed by both ligninolytic peroxidases, produced simultaneously.

Finally, despite some differences, the degradation of the PAHs followed the same scheme, forming quinone metabolites at the first stage: 9,10-anthraquinone in the case of anthracene, 9-fluorenone in the case of fluorene, and phenanthrene-9,10-quinone in the case of phenanthrene. The presence in the cultivation medium of only laccase (Str. rugosomannulata and A. bisporus) resulted in accumulation of the corresponding quinones in the cultivation medium. Successive production of laccase and Mn-peroxidase (P. ostreatus) resulted in the formation and subsequent utilization of these metabolites. Finally, if both enzymes were highly active (T. versicolor DSM), the quinone metabolites formed were degraded quickly.

The obtained data supported our hypothesis that, the degree of degradation of the PAHs can depend on the composition of the extracellular ligninolytic complex. Earlier, we showed that the completeness of degradation of fluorene, fluoranthene, pyrene, and chrysene by P. ostreatus D1 depends on the cultivation conditions. For example, in Kirk’s medium about 65.6 % of the initial pyrene was metabolized after 3 weeks, with pyrene-4,5-dihydrodiol accumulating. This process was accompanied by laccase production only. In the basidiomycetes rich medium, P. ostreatus D1 metabolized up to 89.8% of pyrene within 3 weeks without pyrene-4,5-dihydrodiol accumulation. Phenanthric and phthalic acids were identified as the metabolites. Accumulation of phenanthric acid, with its subsequent disappearance, was observed. Pyrene metabolism in the basidiomycetes rich medium was accompanied first by laccase production and later by versatile peroxidase production. The data indicate that both enzymes (laccase and versatile peroxidase) are necessary for the complete degradation of pyrene [28]. This effect was confirmed for the other PAHs: chrysene, fluorene, and fluoranthene. Under the laccase production conditions, transformation of chrysene by P. ostreatus D1 occurs with accumulation of the quinone metabolite. Under conditions of production of both laccase and versatile peroxidases, chrysene degradation occurs, leading to phthalic acid formation and its further utilization [29]. Fluorene and fluoranthene were transformed in Kirk’s medium (under conditions of laccase production), with the formation of a quinone metabolite and 9-fluorenone, when fluoranthene and fluorene were used as the substrates, respectively. More complete degradation with the formation of an intermediate metabolite, phthalic acid, which underwent subsequent utilization, occurred in the basidiomycetes rich medium (under the production of both laccase and versatile peroxidase) [30].

Therefore, the affiliation of the fungi with different ecophysiological groups and their cultivation conditions affect the composition and dynamics of production of the ligninolytic enzyme complex and, consequently, the completeness of PAH utilization. Comparison of the obtained data permits us to suppose that laccase can catalyze the initial attack on the PAH molecules, which results in quinone formation. It should be noted that the quinone derivatives are highly toxic, as we showed earlier with Sorghum bicolor L. Moench and Medicago sativa L. seedlings [31]. Perhaps peroxidases catalyze the following oxidation of these compounds, resulting in pollutant mineralization. In addition, it is necessary to investigate the role of different enzymes and factors in fungal degradation of the PAHs.

In this regard, for the correct selection of fungal strains for mycoremediation, it is necessary not only to identify the metabolites formed, but also to study the activity of the basic ligninolytic enzymes. This will allow the development of a technological process to avoid the accumulation of toxic substances in the treated objects. On the basis of their degradative properties and the composition of
the ligninolytic enzyme system, the fungi *Trametes* and *Pleurotus* can be chosen for detailed study and for the development of technologies of mycoremediation of PAH-contaminated environments.

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