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Degradation of Polycyclic Aromatic Hydrocarbons by Selected White-rot Fungi and the Influence of Lignin Peroxidase

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Abstract The white-rot fungi *Phanerochaete chrysosporium* ATCC 24725, *Pleurotus ostreatus* ATCC 32783, *Lentinus edodes* ATCC 24462, and *Trametes versicolor* ATCC 42530 were studied for their ability to degrade lignin, phenanthrene, and anthracene. Lignin in rice-straw was degraded by 14.4, 28.73, and 33.88% by *P. chrysosporium*, *T. versicolor*, and *P. ostreatus*, respectively. Approximately 12% and 83% of phenanthrene was degraded in 1 and 5 days, respectively, when the pre-grown mycellium matrix of *P. ostreatus* was incubated with 10 ppm of phenanthrene in modified Kirk's medium (nitrogen limited) at 25°C. Approximately 2% and 61% of phenanthrene was degraded when the phenanthrene concentration was increased to 30 ppm. Similar trends were observed with phenanthrene using *P. chrysosporium*. Mycelial growth of *T. versicolor* was less inhibited at 30 ppm phenanthrene than for *P. ostreatus* and *P. chrysosporium*. Better degradation of phenanthrene by *T. versicolor* may be attributed to better mycelium growth. One hundred percent of 15 ppm anthracene was degraded in 10 days by both *P. chrysosporium* and *T. versicolor*. 40 ppm anthracene inhibited the mycelial growth of *P. chrysosporium*. Lignin peroxidase activity, which was previously reported to be involved in initial phenanthrene oxidation, was also detected from the culture broth of the strains tested. The rates of lignin peroxidase production in the cultures were not consistant with the rate of PAH hydrolysis during incubation.

Key words: White-rot fungi, phenanthrene, anthracene, lignin, bioremediation

Lignin is an aromatic polymer forming up to 30% of woody plant tissues, providing rigidity and resistance to biological attack. Lignin is a difficult substrate for enzyme degradation because it is insoluble, chemically complex, and lacks hydrolyzable linkages [18]. The most effective lignin degraders in nature are the white-rot fungi which belong to the Basidiomycetes [2, 16].

Polycyclic aromatic hydrocarbons (PAHs), such as phenanthrene and anthracene, are ubiquitous pollutants originating from pyrolysis of organic matter and processes of the oil industry. PAHs and other chlorinated hydrocarbons persist in the environment, eventually accumulating in the body fat of animals including humans through the aquatic and terrestrial ecosystems. Of concern to public health is the fact that many PAHs and their metabolites are mutagenic and/or carcinogenic. The high cost of trapping, incineration, and removal of PAHs from the environment has increased interest in the use of microorganisms for the biological decontamination of PAH-polluted sites [1, 6].

White-rot fungi can completely mineralize lignin and a wide variety of environmental pollutants, such as PAHs and PCBs [4, 5, 7, 8, 11, 12]. The degadation of both lignin and pollutants by these fungi depends on the production and secretion of lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase, which are the key components of lignin degrading enzyme systems [17]. The potential of white-rot fungi for *in situ* bioremediation has been attributed to their ability to degrade a variety of xenobiotic chemicals via a free radical mechanism mediated by extracellular peroxidase. LiP is of particular interest for pollutant degradation and is produced in response to limited nutrients, such as nitrogen, carbon, and sulfur. Consequently, N-limited conditions are typically used to study the production of peroxidase [20] and the degradation of environmental pollutants [3, 10, 14]. Research results have suggested that lignin peroxidase is the prime candidate for catalysis of initial PAH oxidation [17]. However, thus far, no conclusive evidence for involvement of the lignin

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peroxidase system of white-rot fungi, including *P. chrysosporium*, in PAH metabolism has been presented [12, 13, 15].

The objective of this study was to evaluate selected white-rot fungi strains, including *P. chrysosporium*, *T. versicolor*, *L. edodes*, and *P. ostreatus*, for degradation of the 3-ring PAHs, phenanthrene and anthracene. This study also examined reaction parameters in order to characterize PAH degradation by the selected white-rot fungi. Results will be applied to bioremediation of contaminated soils and sediments.

MATERIALS AND METHODS

Organisms

White-rot fungi used were *Pleurotus ostreatus* ATCC 32783, *Lentinus edodes* ATCC 24462, *Trametes versicolor* ATCC 42530, and *Phanerochaete chrysosporium* ATCC 24725. These were purchased from the American Type Culture Collection (ATCC).

Medium and Culture Conditions

Stock cultures were prepared by growing the organisms on potato dextrose agar (PDA) slants. Table 1 summarizes the culture conditions for the selected white-rot fungi. All cultures were stored at 4°C. Cultures for lignin peroxidase production were grown in a liquid medium derived from that of Tien and Kirk [20] with some modification (Table 2).

Spores of *P. chrysosporium* from the PDA plates were collected and filtered through sterile glass wool to remove the mycelial debris. 5×10^7 spores were inoculated into a 1 *l*-Erlenmyer flask containing 50 ml of modified Kirk's medium under nitrogen limited conditions. The flasks were incubated at 37°C for 48 h without agitation. After 48 h the mycelial mat was washed twice with sterilized water and homogenized for 10 sec in a blender, and then 6~7 mg/ml (mg-dry cell/ml) was transferred to a sterile 125 ml-serum bottle containing 10 ml of modified Kirk's medium. Phenanthrene and anthracene were added to the medium after being dissolved in *N,N'*-dimethyl formamide. All serum bottles were incubated at 37°C without agitation and flushed with oxygen every 2 or 3 days. The degradation profiles of phenanthrene and anthracene were analyzed from 0 to 30 days.

The inoculum of *P. ostreatus*, *L. edodes*, and *T. versicolor* was prepared with a mycelial mat which was cultured for 6 to 14 days without agitation, and then washed twice with autoclaved water. The mats were homogenized in a blender for 10 sec. The rest of the procedure was the same as for *P. chrysosporium*, except that incubation was at 25°C.

Table 1. Culture conditions for selected white-rot fungi.

Strain Type	Temperature (°C)	Incubation Time (day)
Pleurotus ostreatus (ATCC* 32783)	25	7
Lentinus edodes (ATCC 24462)	25	10
Trametes versicolor (ATCC 42530)	25	5
Phanerochaete chrysosporium (ATCC 24725)	37	3

* American Type Culture Collection

Table 2. Composition of culture media for white-fungi.

Media composition (shallow stationary cultures)

The following items are added per *l* of shallow stationary cultures;
- [1]Basal III medium (autoclaved), 100 ml
- 10% glucose (autoclaved), 100 ml
- 0.1 mM trans-aconitic acid (autoclaved), 100 mM
- Thiamin (100 mg/*l* stock; autoclaved), 10 ml
- Ammonium tartrate (8 g/*l* stock; autoclaved), 20 ml
- Veratryl alcohol (0.1 M stock; filter sterilized), 25 ml

[1]Basal III medium (/liter);
- KH_2PO_4, 20 g
- $MgSO_4$, 5 g
- $CaCl_2$, 1 g
- *Trace element solution, 100 ml

*Trace element solution (/liter);
- $MgSO_4$, 3 g
- $MnSO_4$, 0.5 g
- NaCl, 1.0 g
- $FeSO_4 \cdot 7H_2O$, 0.1 g
- $CoCl_2$, 0.1 g
- $ZnSO_4 \cdot 7H_2O$, 0.1 g
- $CuSO_4$, 0.1 g
- $AlK(SO_4)_2 \cdot 12\ H_2O$, 10 mg
- H_3BO_3, 10 mg
- $NaMoO_4 \cdot 2\ H_2O$, 10 mg
- **Nitrilotriacetate, 1.5 g

**Dissolve nitrilotriacetate in 800 ml H_2O, adjust pH to 6.5 with 1 N KOH, add each component, and then bring the volume to 1 liter.

HPLC

For analysis of phenanthrene degradadation the culture broth and mycelia were extracted with 5 volumes of

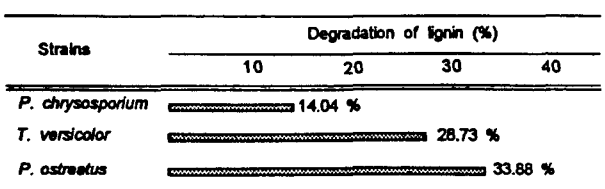

Fig. 1. Degradation of lignin in rice-straw by white-rot fungi. *P. chrysosporium* was incubated at 37°C for 30 days and *T. versicolor* and *P. ostreatus* were incubated at 25°C for 30 days.

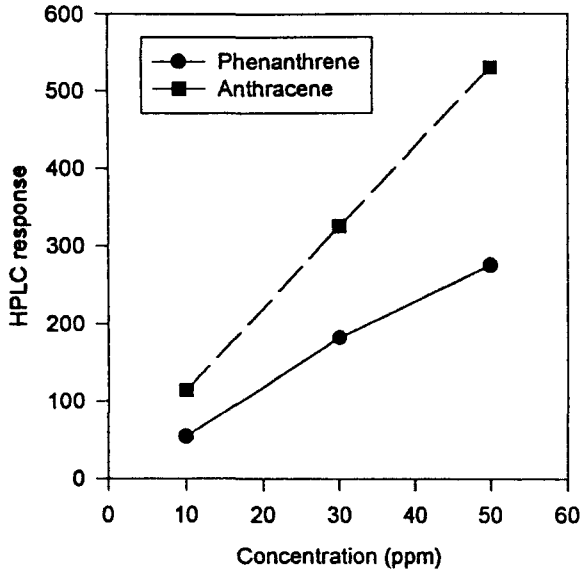

Fig. 2. Standard curve of phenanthrene and anthracene.

ethylacetate [9]. The extracts were dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure. Reverse-phase high performance liquid chromatography analysis of phenanthrene was performed with a Beckman model 420 liquid chromatograph fitted with a Beckman Ultrasphere C18 column. The mobile phase consisted of a methanol-water mixture (75:25, v/v). Analysis was done for 30 min with a flow rate of 1 ml/min. A Beckman model 166 UV absorbance detector was set on 254 nm to detect phenanthrene. Anthracene was analyzed by the same method. The concentrations of phenanthrene and anthracene were standardized by the results shown at Fig. 2.

Lignin Peroxidase Activity

Lignin peroxidase activity was estimated as the H_2O_2-dependent oxidation rate of veratryl alcohol to veratryl aldehyde (extinction coefficient 9300 $M^{-1}cm^{-1}$) using the culture supernatant at 310 nm [20]. The reaction mixture contained 180 µl of culture broth, a 50 mM sodium tartarate buffer (pH 3.0), 2 mM veratryl alcohol, and 0.4 mM H_2O_2 in a final volume of 1 ml. The activity was reported as units per liter. One unit was defined as the amount of enzyme required to oxidize one µmole of veratryl alcohol per min.

Degradation of Lignin

Lignin in rice-straw was used. White-rot fungi were inoculated into a 125 ml-Erlenmyer flask containing 5 ml of 10 mM trans-aconitic acid and 2 g of ball-milled rice-straw (100 mesh) autoclaved. The flasks were incubated at 37°C for *P. chrysosporium* and 25°C for *P. ostreatus, L. edodes*, and *T. versicolor* and flushed with oxygen for 5 min every 5 days. The degradation rate of lignin in rice-straw was analyzed after 30 days of incubation using a modified Klason method [21].

Results and Discussion

Degradation of Lignin in Rice-straw

Since white-rot fungi are unable to use lignin and other polycyclic aromatic hydrocarbons (PAHs) as growth substrates, glucose was added to the ball-milled rice-straw. *L. edodes* was not included in this experiment due to its slow growth rate under the experimental conditions. *P. ostreatus* showed the best degradation of lignin among the three strains tested. Approximately 14% and 34% of lignin in rice-straw were degraded by *P. chrysosporium* and *P. ostreatus*, respectively (Fig. 1). Nishida *et al.* [16] observed that 11% and 24% of beech wood lignin were degraded by *P. chrysosporium* ME 446 and *Coriolus versicolor*, respectively, under the same experimental conditions. The use of *T. versicolor* and *P. ostreatus* has been studied in paper pulp and animal feed production due to their excellent lignin degrading activities in wood and in most agricultural by-products, such as cereal straws. Because of the structural resemblance of lignin to PAHs, lignin degrading white-rot fungi can be used for PAH degradation in bioremediation and for biopulping to save the cost of mechanical grinding and to increase the degradability of forage.

Degradation of Phenanthrene

No inhibitory effect on the growth of the fungi was observed at 10 ppm phenanthrene in the culture broth, while the growth of fungi mycelia stopped completely at 50 ppm phenanthrene. Compared to *P. ostreatus*, growth of *T. versicolor* was less inhibited at 30 ppm phenanthrene and the fungi tested in this experiment did not grow at 50 ppm phenanthrene. The better degradation rate of *T. versicolor* at 30 ppm phenanthrene in the culture broth (Fig. 3) may be attributed to better growth of mycelia. No growth of the fungi at 50 ppm phenanthrene resulted in a minimum degradation of phenanthrene. *T. versicolor* and *P. ostreatus* are better candidates for the *in vitro* phenanthrene degradation at concentrations lower than 50 ppm.

Lignin peroxidase (LiP) activities in the culture broth of *P. chrysosporium* were determined during 30 days incubation in the presence of 15 ppm phenanthrene (Table 3). The maximum LiP activity was 129.43 U/l at 5 days incubation at which 48% of added phenanthrene was degraded. However, the degradation of phenanthrene reached the maximum level at 75% when LiP activity was 35 U/*l* after 30 days incubation. As previously shown in the results by Hammel *et al.* [12], the level of lignin peroxidase is apparently unrelated to the rate of phenanthrene degradation.

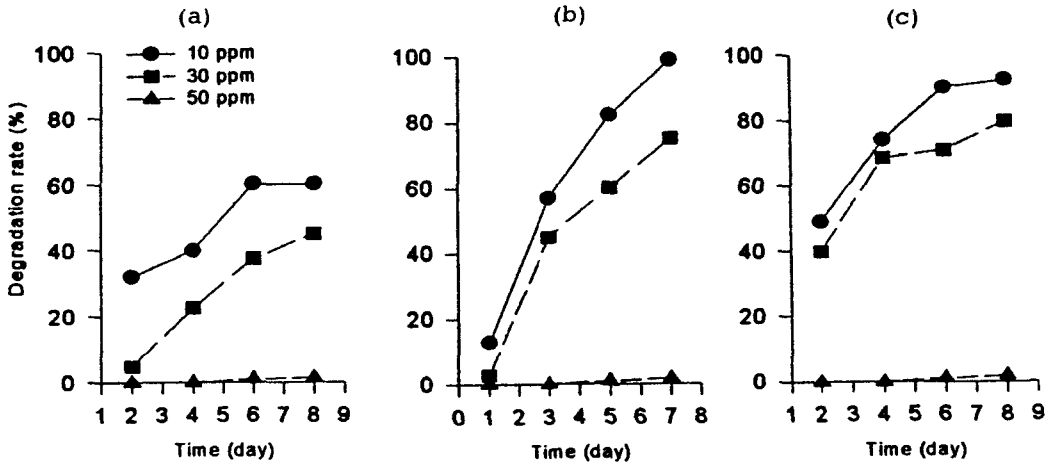

Fig. 3. Degradation rate of phenanthrene by *P. chrysosporium* (a) at 37°C, and *T. versicolor* (b) and *P. ostreatus* (c) at 25°C.

Table 3. Changes of various factors in culture broth of *P. chrysosporium* of 15 ppm phenanthrene during incubation at 37°C.

Incubation time (days)	Degradation of phenanthrene (%)	Lignin peroxidase activity (U/l)	Degradation of glucose (%)	pH
5	48	129.43	59	4.8
10	55	71.93	93	5.8
20	71	37.31	100	6.2
30	75	35.22	100	6.2

All added glucose was consumed between incubation days 10 and 20, and the pH increased to 6.2 during incubation. When residual glucose was not present in the culture broth from days 20 to 30, phenanthrene degradadation was limited to between 71% and 75%. The degradation rate of phenanthrene apparently was more influenced by the glucose concentration and the culture broth pH. We observed results similar to other investigators in experiments with different substrates [3].

Degradation of Anthracene

Degradation of 15 ppm anthracene by *P. chrysosporium* was faster than degradation of phenanthrene. Only 55% of phenanthrene was degraded in 10 days while almost all added anthracene was degraded over the same incubation period (Table 4). The mycelium of *T. versicolor* degraded 100% of 15 ppm anthracene in 10 days and degraded 100% of 10 ppm phenanthrene in a week. Only 54% of 15 ppm anthracene was degraded by *P. ostreatus* in 10 days, but 93% of 10 ppm phenanthrene was degraded over the same incubation period. Comparing the degradation of phenanthrene and anthracene, *P. chrysosporium* was a more efficient degrader of anthracene, but *P. ostreatus* was better able to degrade phenanthrene (Fig. 3 and Table 4). The concentration of anthracene in the culture medium influenced the degradation rate (Table 5). Ninety percent of anthracene was degraded in 5 days when 15 ppm anthracene was added, whereas only 10.4% was degraded when 50 ppm anthracene was added to the *P. chrysosporium* culture. The growth of fungi mycelia also stopped at 50 ppm anthracene in the culture broth, which was the same

Table 4. Changes of various factors during degradation of 15 ppm anthracene by white-rot fungi; *P. chrysosporium*, *T. versicolor*, and *P. ostreatus*.

Strain	Time (days)	Degradation of anthracene (%)	Lignin peoxidase activity (U/l)	Degradation of glucose (%)	pH
P. chrysosporium	5	90.2	41.2	60.2	N.D.*
	10	99.9	40.4	96.3	4.7
T. versicolor	5	73.0	23.5	46.6	N.D.
	10	100.0	22.5	68.5	4.3
P. ostreatus	5	24.5	1027.1	24.0	4.7
	10	54.2	563.7	25.0	N.D.
	20	91.4	378.2	39.0	4.3

* N.D.: Not determined.

Table 5. Effect of concentration on the anthracene degradation using *P. chrysosporium* mycelium at 37°C for 5 days.

Anth. conc. (ppm)	Degradation (%)	
	fresh & added*	heat-treated & added**
15	90.0	2.5
20	69.8	2.1
30	60.9	2.5
40	14.1	3.3
50	10.4	3.7

*Mycellium of *P. chrysosporium* was prepared as described in Materials and Methods.
**Mycellium of *P. chrysosporium* was heat-treated to remove the viability.

trend as in the phenanthrene experiment. It may cause the limited degradation of 50 ppm anthracene by the *P. chrysosporium* culture. The *P. chrysosporium* adsorption of anthracene ranged from 2.1% to 3.7% which was not of great concern in the measurement of the degradation rate. *T. versicolor* had a similar effect on the degradation of both phenanthrene and anthracene.

The LiP activity of *P. ostreatus* in the culture broth was higher than for *P. chrysosporium* and *T. versicolor*. The culture broth of *P. ostreatus* showed the highest LiP activity at day 5 among the three strains. It was 25 and 44 times higher than the activities of *P. chrysosporium* and *T. versicolor*, respectively (Table 4). However, degradation of anthracene by *P. ostreatus* was the lowest compared to the other strains. The pattern of glucose consumption was also different among strains tested; 96% of glucose was used by *P. chrysosporium*, but only 39% of added glucose was used by *P. ostreatus* after 20 days of incubation.

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