

Biodegradation of Aromatic Hydrocarbons by Several White-rot Fungi

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To investigate the biodegrading capability of several white-rot fungi isolated in Korea, biodegradation of BTX (benzene, toluene, xylene), phenanthrene and pyrene were tested in fungal cultures. *Phanerochaete chrysosporium* removed 20-30% of BTX mixture during 21 days of incubation in serum bottle. *Coriolus versicolor* KR-11W and *Irpex lacteus* mineralized 10.02 and 8.26% of total phenanthrene, respectively, which were higher than in other studies with *P. chrysosporium*. These two strains also showed high mineralization rates (9.2-10.1%) for 4-ring pyrene. *I. lacteus* metabolized most of the added pyrene and 23.29% was incorporated into fungal biomass. Almost 50% of the pyrene was converted to polar metabolites and recovered from aqueous phase of culture. These results indicated that some white-rot fungi have higher biodegradability than *P. chrysosporium* and could be used in bioremediation of aromatic hydrocarbon contaminants in soil.

Key words: biodegradation, BTX, phenanthrene, pyrene, white-rot fungi

White-rot fungi are wood decomposing basidiomycetes that are capable of degrading not only lignin, but also a variety of relatively recalcitrant environmental pollutants. These contaminants include some aromatic hydrocarbons and are broken down using lignin peroxidase, manganese peroxidase, laccase and other enzymes (8, 12). Aromatic hydrocarbons are common environmental pollutants that arise from industrial operations and from natural events like forest fires (9). They are major component in petroleum and are continuously released into natural environments. Most aromatic compounds are toxic to living organisms, and some of them and/or their metabolites are carcinogenic. There have been numerous studies on the biodegradation and bioremediation of many aromatic hydrocarbons, most of them focused on the degrading bacteria. However, white-rot fungi are likely to be involved in the biodegradation of persistent pollutants in soil because they can degrade recalcitrant lignin and humic compounds that are random polymers of aromatic structures. Moreover, they are indigenous soil microorganisms and widely distributed. Recently their use in polycyclic aromatic hydrocarbons (PAHs) bioremediation has been proposed (17). To date, most attention has been directed at *Phanerochaete chrysosporium* (4, 5, 6, 7, 8, 23, 25). Only a few reports have been published on other white-rot fungi (2, 21, 26), and other groups of fungi (15, 16), although the possibility exists that some other fungal species may be more

effective than *P. chrysosporium* in biodegradation of aromatic hydrocarbons like PAH pollutants. This report compares several white-rot fungal strains isolated in Korea, which already showed a high decolorizing activity of dyes (14), as to their relative ability to degrade BTX mixture, 3-ring PAH phenanthrene, and 4-ring PAH pyrene in culture solution. This work was performed as a preliminary assessment of their potential usefulness in the bioremediation of aromatic hydrocarbon pollutants.

Materials and Methods

Preparation of fungal inoculum

Phanerochaete chrysosporium IFO 31249, *Pleurotus ostreatus*, and *Microporus vernicipes* were obtained from the Mycology Laboratory in the Department of Microbiology at Seoul National University. *Coriolus versicolor* KR-11W, *C. versicolor* KR-65W and *Irpex lacteus* were obtained from the Mycology Laboratory in the Department of Biology at Kangreung National University. All five strains except *P. chrysosporium* IFO 31249, an ATCC strain, were isolated from soil in Korea and identified in those laboratories. The fungi were maintained on YMG medium (yeast extract 4 g, malt extract 10 g, glucose 4 g per 1 L H₂O) solidified with 2% agar. A small portion of a YMG agar plate covered with white-rot fungus was cut out and transferred to YMG broth in an Erlenmeyer flask and was incubated at 28°C on a rotary shaker at 130

rpm for 7 days. Thirty ml of this culture was transferred to a sterile tube and centrifuged (12,000 g, 15 min). The supernatant was decanted into a sterile tube and the fungal pellet was weighed. The pellet was resuspended with the supernatant and the concentration of fungal biomass was adjusted to 1 g wet wt/10 ml culture supernatant. This suspension was blended for 30 to 120 seconds using an Omni-mixer (Ivan Sorvall Inc., Norwalk, Conn.) at 16,000 rpm and the blended fungal culture was used as the inoculum for the biodegradation test.

BTX biodegradation

Sterile YMG broth (10 ml for static culture and 20 ml for shaken culture) in a 125-ml serum bottle (Wheaton) was inoculated with fungal inoculum at 10% (v/v). Each of the inoculated bottles was flushed with pure oxygen for 1 min and sealed with a Mininert valve (Alltech Inc.) which was made of Teflon, preventing the adsorption of hydrocarbons on it. BTX were injected separately into the serum bottle at a concentration of 100 mg/liter (100 ppm) through the valve septum in Mininert valve with a GC syringe. The cultures were incubated at 28°C and shaken cultures were maintained on a rotary shaker (130 rpm). Uninoculated medium controls and heat-killed culture controls were also included. On every 3-4 days, 100 µl of headspace gas was sampled with syringe and analyzed using a gas chromatograph equipped with a flame ionization detector (Hewlett-Packard Co., Model 5890). The operating conditions are described elsewhere (22).

Biodegradation of radioactive phenanthrene and pyrene

The fungal suspension (2.5 ml) was inoculated into 25 ml of sterile YMG broth in modified Fernbach flasks (18). To provide aerobic conditions between trapping times, the flask headspace (250 ml) was flushed with pure oxygen instead of air. After flushing for 1 min with oxygen, the flasks were sealed and 1.39 nmol of [9,10-¹⁴C] phenanthrene (52.98 mCi/mmol) or 0.84 nmol of [4,5,9,10-¹⁴C] pyrene (55 mCi/mmol, Chemsyn Science Lab.) in 2.5 µl of benzene was injected into each flask through the syringe port. The fungal cultures were incubated at 28°C. The CO₂ and the volatile compounds in headspace evolved from degradation were trapped periodically with a gas-trapping device (18) providing pure oxygen for flushing. The ¹⁴CO₂ trapped in Oxosol C14 (National Diagnostics, Atlanta, GA) and the volatiles trapped in Scintiverse BD cocktail (Fischer Scientific, Pittsburgh, PA) were quantified by liquid scintillation counter (Beta-Trac model 6895, Tm Analytic, Elk Grove Village, IL).

Corrections were made for background and for counting efficiency using the external standard ratio method.

Analysis of the radiolabel remaining after pyrene biodegradation

At the end of incubation period (21 days), the cultures were filtered through pre-weighed filter paper (Whatman No. 5) and air-dried, and the mycelial dry weights were determined. The fungal biomass was Soxhlet-extracted for 6 hours using methylene chloride. The methylene chloride extract was concentrated to 10 ml with a rotary evaporator, and 1 ml was put into Scintiverse BD cocktail and its radioactivity was determined. After Soxhlet extraction, the radioactivity in remaining biomass was detected by wet combustion (1) and trapping of the evolved ¹⁴CO₂ in Oxosol cocktail. The filtrates of the fungal cultures were extracted with methylene chloride three times using a separatory funnel, and each phase was collected separately. Usually an emulsion layer formed between the aqueous and the solvent phase. To break it, the emulsion was passed through an anhydrous sodium sulfate layer. This sodium sulfate was subsequently air-dried, wet-combusted, and its radioactivity was determined. The radioactivity of the aqueous phases and of the methylene chloride extracts of the culture filtrates was counted using Scintiverse BD cocktail. The methylene chloride extracts of filtered biomass and culture fluid were analyzed using gas chromatograph. The operating conditions were described elsewhere (22).

Results and Discussion

Biodegradation of BTX

To make a closed system for volatile BTX mixture and provide aerobic conditions for fungal metabolism, a serum bottle with Mininert valve was used in this BTX experiment. In this experiment only *Phanerochaete chrysosporium* was tested and it removed 15.5, 22.1, and 30.1% of benzene, toluene, and *p*-xylene, respectively during 21 days of period in static culture (Fig. 1). In shaken culture 22.8, 19.1, and 25.5% of BTX were removed by fungus (Fig. 2). The removal rates of benzene were higher than those in many conditions and media in a similar study (27). The removal rates of toluene and *p*-xylene were higher than those with basal medium, but lower than their results (40% of toluene and 60% of *p*-xylene) with ME medium (27). However, the concentration of BTX in this experiment was 10-fold higher, so it could have affected biodegradability. The biodegradation rates by *P. chrysos-*

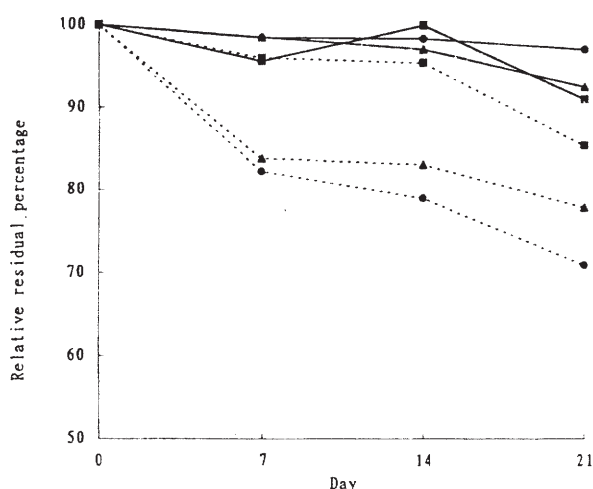


Fig. 1. Disappearance of BTX from headspace of static culture of *Phanerochaete chrysosporium*. -■-: Benzene (control), -■-: Benzene (culture), -▲-: Toluene (control), -▲-: Toluene (culture), -●-: Xylene (control), -●-: Xylene (culture).

porium were lower than expected, and it might be from the difficulty of maintaining of aerobic culture conditions in a small serum bottle (actual volume: 160 ml). The white-rot fungus is an obligately aerobic organism, but it was hard to maintain a closed system for volatile BTX and provide enough oxygen to fungus simultaneously. The toxicity of, and substrate interactions among BTX mixture, such as competitive inhibition and cometabolism (10, 19) seemed to be another reason for low biodegradation rates. A higher biodegradation rate might be expected if aerobic condition and lower concentrations of BTX were maintained.

Biodegradation of radioactive phenanthrene

Since phenanthrene and pyrene are not volatile aromatics, biodegradation experiments of phenanthrene and pyrene were carried out on modified Fernbach flasks (18) which could be supplied with oxygen while trapping the headspace gas. *Coriolus versicolor* KR-11W produced 10.02% of $^{14}\text{CO}_2$ during 21 days, which was the highest mineralization rate and *Irpex lacteus* formed 8.26% of $^{14}\text{CO}_2$ (Fig. 3). *P. chrysosporium* and *Pleurotus ostreatus* showed lower mineralization rates. *C. versicolor* KR-65W and *Microporus vernicipes* produced only a trace amount of $^{14}\text{CO}_2$. The cultures of these two strains turned dark during the incubation, indicating the formation of melanin type pigments. The ligninolytic enzymes are known to be involved in pigment production (11, 24) and co-polymerization of lignin (20). In these two strains, most ligninolytic enzymes seemed to be involved in pigment formation and did not participate in the biodegradation of phenan-

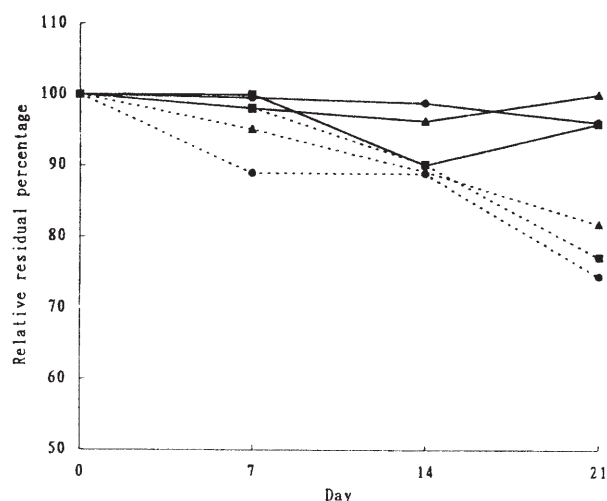


Fig. 2. Disappearance of BTX from headspace of shaken culture of *Phanerochaete chrysosporium*. -■-: Benzene (control), -■-: Benzene (culture), -▲-: Toluene (control), -▲-: Toluene (culture), -●-: Xylene (control), -●-: Xylene (culture).

threne. The other four strains tested, which evolved $^{14}\text{CO}_2$, did not produce the dark pigments. The $^{14}\text{CO}_2$ evolution from phenanthrene by *C. versicolor* KR-11W and *I. lacteus* was much higher than that in other studies with *P. chrysosporium* (2, 4, 7), but *P. chrysosporium* showed similar results to them in this experiment. This indicates that *C. versicolor* KR-11W and *I. lacteus* have high biodegrading capability and can be candidates for bioremediation. During the metabolism of phenanthrene, some volatile metabolic intermediates were produced (Fig.

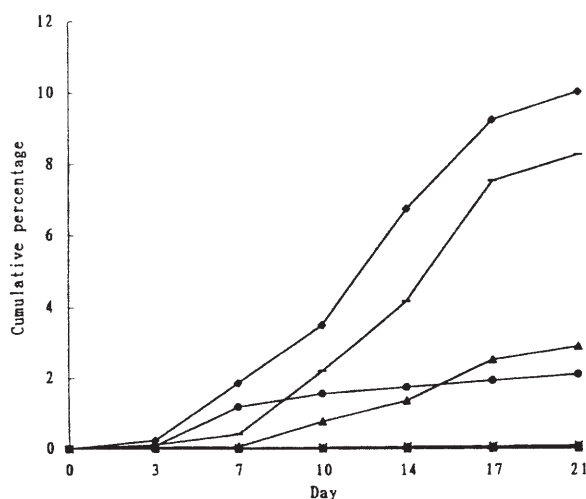


Fig. 3. Evolution of radioactive carbon dioxide from phenanthrene biodegradation by white-rot fungi. -▲-: *Phanerochaete chrysosporium*, -●-: *Pleurotus ostreatus*, -◆-: *Coriolus versicolor* KR-11W, -×-: *Coriolus versicolor* KR-65W, —: *Irpex lacteus*, -+ -: *Microporus vernicipes*, -■-: Control.

4). The amounts of volatiles were much less than $^{14}\text{CO}_2$, ranging from 0.7 to 1.76% of original substrate. All fungal strains produced volatile intermediates including *C. versicolor* KR-65W and *M. vernicipes* which showed very low mineralization. Even though complete mineralization was not caused by some fungi, those metabolic intermediates could be utilized by other organisms in natural environments.

Biodegradation of radioactive pyrene

The time course and extent of pyrene mineralization by three white-rot fungi are shown in Fig. 5. *P. chrysosporium*, the species studied in the greatest detail to date, converted 5.8% of the added pyrene to $^{14}\text{CO}_2$. *C. versicolor* KR-11W and *I. lacteus* showed conversions that were considerably higher at 9.2 and 10.1%, respectively. Although lower than those reported for some bacteria (13), these conversions were higher than those with other fungi (2, 4, 7, 16). The two highly active strains tested were new isolates, but other strains of the same species were also used as part of the mixed inoculum used in the bioaugmentation experiments of Lestan and Lamar (17). The mineralization of pyrene was max-

imal between days 7 and 17. During the first days of the experiment biomass was relatively low. Past day 17, degradation activity may have been limited by nutrient depletion and culture senescence. Prior to the $^{14}\text{CO}_2$ traps, the headspace gas passed through traps filled with Scintiverse BD counting fluid, designed to trap non- CO_2 volatiles. From uninoculated control flasks, only 0.10% of the total radioactivity was volatilized, most likely in form of intact pyrene. From the fungal cultures, 0.11-0.13% of the added radioactivity was trapped in Scintiverse, showing that essentially no volatiles other than $^{14}\text{CO}_2$ were produced. The results reflect the low volatility of pyrene. They also contrast with the results of Bogan and Lamar (4) who obtained more volatiles from several PAH compounds. The mineralization of pyrene was slightly higher than that of phenanthrene, which was expected to be more easily biodegradable. However, this kind of result can be seen in other study (4), and the fractionation of phenanthrene culture should be carried on to examine the total metabolic transformation described below.

Fate of radiolabeled pyrene during biodegradation

After 3 weeks of incubation, the fungal culture of *I. lacteus* that showed the highest mineralization

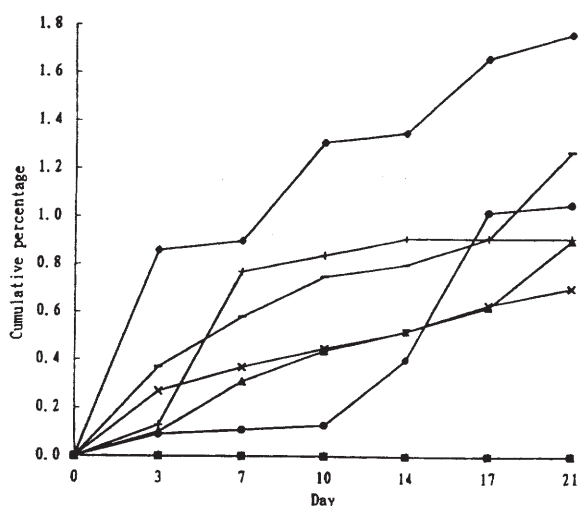


Fig. 4. Evolution of radioactive volatile compounds from phenanthrene during biodegradation by white-rot fungi. Δ —: *Phanerochaete chrysosporium*, \bullet —: *Pleurotus ostreatus*, \blacktriangle —: *Coriolus versicolor* KR-11W, \times —: *Coriolus versicolor* KR-65W, —: *Irpex lacteus*, $+ +$ —: *Microporus vernicipes*, \blacksquare —: Control.

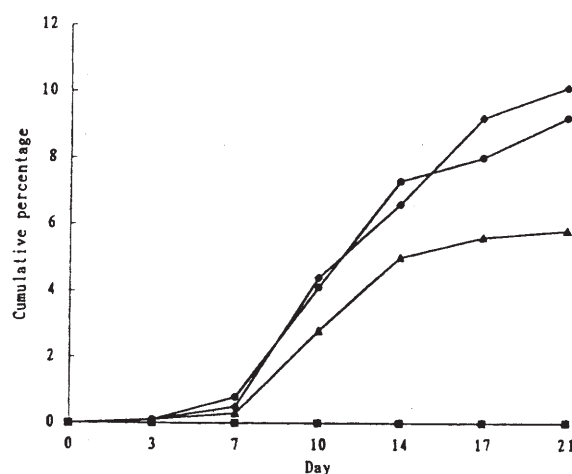


Fig. 5. Evolution of radioactive carbon dioxide from pyrene biodegradation by white-rot fungi. \blacksquare —: Control, Δ —: *Phanerochaete chrysosporium*, \bullet —: *Coriolus versicolor* KR-11W, \blacklozenge —: *Irpex lacteus*.

Table 1. Fate of pyrene during biodegradation by *Irpex lacteus* (unit: residual percentage)

CO_2 evolved	non- CO_2 volatiles	biomass incorporation	Soxhlet extraction of filtered biomass	solvent extraction of culture filtrate	emulsified components	aqueous phase
10.1	0.12	23.29	1.23	1.31	6.80	42.65

rate was separated into several fractions. As shown in Table 1, most of the residual radioactivity was present in the aqueous phase, which seemed to contain polar metabolic intermediates formed by high biodegradation activity. A large amount of the residual radioactivity (23.29%) was present in the biomass of *I. lacteus*. This percentage was higher than the 4.5-20.3% reported for other PAHs (4). The difference might be due to recovery rates and experimental methods. Only a small amount of radioactivity was recovered in Soxhlet-extract of the biomass, indicating that little if any pyrene was present in precipitated or adsorbed form. Similarly, solvent extraction of the culture supernatants recovered little radioactivity. Pyrene has very low water solubility (0.14 mg/L) and most of its metabolites were too polar to be readily partitioned into the solvent. The radioactivity in the emulsion represented also polar, water-soluble pyrene metabolites. Considering this, almost 50% of the added pyrene was transformed to polar metabolites. It is reasonable to assume that these polar metabolites would be degraded further if growth of the fungi would continue and/or other microorganisms were present (3).

The mineralization rate and the fate of pyrene in *I. lacteus* indicates that these strains can be used for clean up polycyclic aromatic hydrocarbon contaminants. More work is needed to determine the pathways and the metabolites which were shown on chromatograms.

Acknowledgments

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