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Bioremediation of Polycyclic Aromatic Hydrocarbons in Creosote-Contaminated Soil by *Peniophora incarnata* KUC8836

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ABSTRACT Polycyclic aromatic hydrocarbons (PAHs) are present in products made from creosote, coal tar, and asphalt. When wood pile treated with creosote is placed in soil, PAHs can contaminate it. Creosote has been used for wood preservation in the past and is composed of approximately 85% PAHs and 15% phenolic compounds. PAHs cause harmful effects to humans and the environment because of their carcinogenic and mutagenic properties. White rot fungi can degrade not only lignin, but also recalcitrant organic compounds such as PAHs. Among numerous white rot fungi used in previous studies, four species were selected to degrade PAHs in a liquid medium. From this evaluation of the degradation of PAHs by the four fungal isolates, two species were ultimately selected for the highest rates of removal. Following 2 weeks of incubation with *Peniophora incarnata* KUC8836, the degradation rates of phenanthrene, fluoranthene, and pyrene were 86.5%, 77.4%, and 82.6%, respectively. *Mycoaciella bispora* KUC8201 showed the highest degradation rate for anthracene (61.8%). Hence, bioremediation of creosote-contaminated soil with an initial concentration of 229.49 mg kg^{-1} PAHs was carried out using the two selected fungi because they could simultaneously degrade 13 more PAHs than the comparison species. More importantly, isolates of *P. incarnata* KUC8836 were discovered as powerful degraders of PAHs by producing laccase and manganese-dependent peroxidase (MnP), with 1.7- and 1.1-fold higher than the comparison species, respectively. Therefore, the white rot fungus may be proposed for the removal of PAHs and xenobiotic compounds in contaminated environments.

KEYWORDS bioremediation, degradation, *Peniophora incarnata*, polycyclic aromatic hydrocarbons (PAHs), white rot fungi

INTRODUCTION

Creosote has been found in soil from deserted train stations to public parks ever since the creosote-treated wood started to be reused for the construction of public amenities. Creosote-contaminated soil has received attention with regard to the degradation of polycyclic aromatic hydrocarbons (PAHs) due to the use of creosote-treated wood for railroad cross ties and landscape facilities in Korea

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and other countries (Kim et al. 2010). Creosote oil had been used for wood preservation in the past (Sandell and Tuominen 1996). It consists primarily of PAHs and phenol, which are recognized carcinogens and mutagens (Polcaro et al. 2008). This contamination can cause adverse health effects and skin disease and can potentially destroy an ecosystem by direct or indirect inhalation (Peters et al. 1999). The exposure to PAHs can cause DNA damage and to induce tumorigenesis based on their molecular mode of action in cells and tissues from humans and animals (Henkler, Stolpmann, and Luch 2012). More importantly, PAHs should be removed due to their low bioavailability, which is a result of their high hydrophobicity, low water solubility, and strong sorption to the soil matrix and sediments (Wang et al. 2009). The removal of PAHs is essential to the optimal performance of indigenous xenobiotic-degrading microbes involved in the successful bioremediation of soil contaminated with xenobiotic compounds (Lee et al. 2013).

Bioremediation using microorganisms has already been established as a useful method for degrading pollutants (Husain, Husain, and Julshrestha 2009). The majority of white rot fungi are capable of degrading lignin due to their ligninolytic system (Janusz et al. 2013). White rot fungi are active lignin destructors due to ligninolytic abilities conferred by an extracellular enzyme complex containing lignin peroxidase (LiP; EC 1.11.1.14), manganese-dependent peroxidase (MnP; EC 1.11.1.13), and laccase (EC 1.10.3.2). In addition, these ligninolytic enzymes oxidize lignin and organic compounds by a nonspecific radical-based reaction as well (Bamforth and Sigleton 2005). Hence, these fungi have been considered to promote the biodegradation of PAHs, especially PAHs of high molecular weight (Ma et al. 2013). Compared with bacteria, some white rot species are better able to colonize soil and compete with the autochthonous microflora (Novotný et al. 2000). These fungi are involved in the oxidation of the lignin present in wood and of a wide range of xenobiotics with aromatic compound structures, including synthetic dyes and recalcitrant compounds (Choi et. al. 2013; Haritash and Kaushik 2009). In previous studies, surveys of the tolerance to PAHs by white rot fungi isolated from Korea were conducted to select species that maximize the degradation of PAHs. Among these fungi, four species were selected to degrade PAHs. The objective of this study was to evaluate the degradation of PAHs in creosote-contaminated soil by the selected fungi and their ligninolytic enzyme activities.

METHODS

Selection of White Rot Fungi for Degradation of PAHs

White rot fungi were selected in our previous study according to their tolerance to PAHs (Lee et al. 2014). *Peniophora incarnata* KUC8836, *Peniophora nuda* KUC9140, *Phanerochaete sordida* KUC8073, and *Mycoaciella bispora* KUC8201 were used in this study because they were determined to be effective degraders of PAHs (Figure 1). To select the species of fungi optimal for soil bioremediation, degradation of PAHs was performed in 2% malt extract (ME) liquid medium. The concentration of four individual PAHs (phenanthrene, anthracene, fluoranthene, and pyrene) was 50 mg L^{-1} (Lee et al. 2010). An uninoculated abiotic negative-control flask and a positive-control flask containing *Phanerochaete chrysosporium* KCTC6293, an effective degrader of PAHs (Bamforth and Sigleton 2005), were treated similarly. After the addition of the PAH stock solutions, the cultures were incubated on a rotary shaker at 150 rpm (27°C) for 2 weeks. At the end of the incubation period, each sample was extracted three times with 100 ml of methylene chloride (Duksan, Korea) to achieve a final volume of 300 ml. Each extracted sample was concentrated to 10 ml using a vacuum rotary evaporator (Eyela N-1000 series; Tokyo Rikakikai Co. Ltd., Tokyo, Japan). Then, the concentrated samples were analyzed by a gas chromatograph–mass spectrometer (GC-MS; model 7890, Agilent Technologies, Santa Clara, CA, USA). Separation was performed using a DB- 5MS column (0.25 mm in diameter by 30 min in length, film thickness 0.25 mm). The GC-MS analysis was conducted according to the method described by Lee et al. (2010).

Soil Sample and Concentrations of PAHs

Creosote-contaminated soil was collected from the Songchu Train Station located in Kyunggi-do, Korea. Soil samples were passed through a 10-mesh screen to enhance homogeneity. The soil was sterilized at 121°C for 20 min (Jiang, Morgan, and Doyle 2002). Subsequently, these samples were manually mixed with

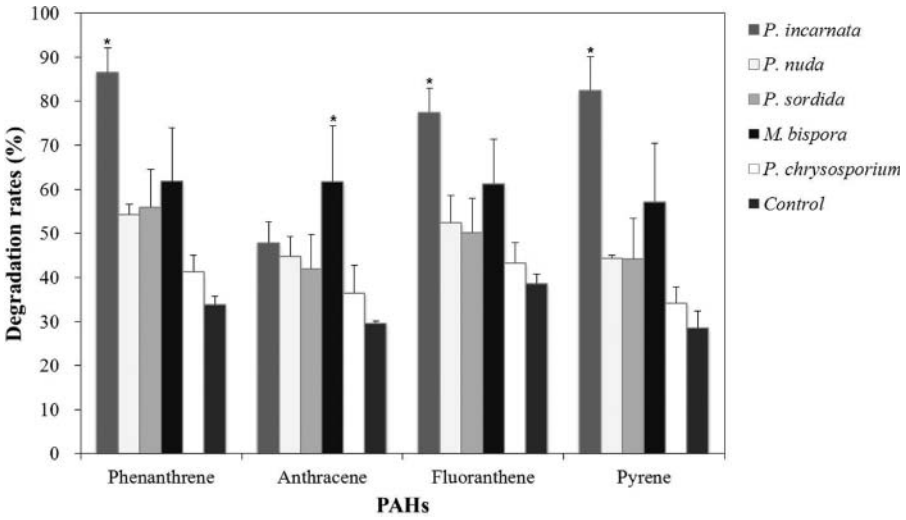

FIGURE 1 **Biodegradation of four PAHs by selected white rot fungi. The asterisk indicates significant difference among the species for each PAH compound ($p < .05$).**

creosote solution containing PAHs and then dissolved in acetone to enhance the concentration of PAHs. For the analysis of PAHs in creosote-contaminated soil, 10 g of soil was extracted in 50 ml of methylene chloride and sonificated by an ultrasonicator at 400 W for 10 min (Lee et al. 2013). Extraction was carried out in five replicates. The extracted samples were filtered using a polytetrafluoroethylene syringe filter (25 mm, 0.45 μm; Whatman) and concentrated to 5 ml. Following this, the PAHs were analyzed in comparison with standard PAHs (NSI Solutions, Raleigh, NC, USA) by GC-MS as described above.

Soil Bioremediation Procedure

Fungal species was primarily cultured in 2% ME agar (MEA) solid medium. Then, five mycelium agar plugs (diameter 5 mm) were obtained from malt agar cultures of each isolate and then inoculated into a 100-ml Erlenmeyer flask containing 50 ml of 2% ME liquid medium. The liquid culture was incubated (150 rpm, 27°C) for 7 days. At the end of incubation, the fungal cultures were homogenized (250 rpm) and centrifuged (4000 rpm, 20 min). Subsequently, the supernatants were removed and mixed with 10 ml of nutrient solution according to the method described by Lee et al. (2013). The fungal stocks were spread throughout a Petri dish (150 × 20 mm) containing 50 g of soil. Then, the samples were incubated in the dark at 27°C for 4 weeks ($n = 5$). For comparison with the selected fungi, *Merulius tremellosus* KUC9161 was used as a biotic control, which is reported to be an effective degrader of PAHs in soil (Lee et al. 2013). The moisture content of the soil was maintained at 60%; organic matter was assessed in accordance with the Walkley-Black procedure (Walkley 1947). In addition, active acidity was measured using 10 g of soil and 50 ml of distilled water, since Wong et al. (2002) reported that PAHs were degraded at a range of pH values (pH 5.5–7.5) with *Burkholderia cocovenenas*–degrading petroleum-contaminated soil.

Ligninolytic Enzyme Assays

Ten grams of soil from each samples was mixed with 20 ml of 0.1 M potassium phosphate (pH 7.0) in an Erlenmeyer flask and incubated for 60 min (100 rpm, 35°C). Then, 5 ml of the supernatant was centrifuged at 4000 rpm for 20 min, and mycelia were removed by a hydrophilic syringe filter (0.45 μm; Sartorius Stedim Biotech, Goettingen, Germany). The filtrate was then used to assay for the activity of the three ligninolytic enzymes, laccase, manganese-dependent peroxidase (MnP), and lignin peroxidase (LiP), using a ultraviolet-visible (UV-Vis) spectrophotometer ($n = 3$). For laccase, 0.1 M sodium acetate (pH 4.5) and 1.5 mM 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were used (Novotný et al. 2004). For MnP, 0.5 M sodium malonate (pH 4.5), 5 mM $MnSO_4$, 1 mM 2,6-dimethoxylphenol, and 1 mM H_2O_2 were used (Wang et al. 2008). For LiP, 0.25 M sodium tartrate (pH 2.5), 10 mM veratryl alcohol, and 5 mM H_2O_2 were used (Tien and Kirk 1988). One unit of ligninolytic enzyme activity produced 1 μmol of reaction

product per minute at room temperature; activity was expressed in U/ml (Hadibarata, Tachibana, and Itoh 2009; Lee et al. 2010).

Data Analysis

The degradation data were analyzed using a Statistical Analysis Systems (SAS 9.2; SAS Institute, Cary, NC, USA) software package. A one-way analysis of variance (ANOVA) followed by a Tukey test was used to assess the significance of degradation between the species. The mean values were compared using a significance level of $p < .05$.

RESULTS AND DISCUSSION

Selection of the Species for Degradation of PAHs

Four species exhibited a high tolerance to both individually and in a mixture four PAHs (phenanthrene, anthracene, fluoranthene, and pyrene), as indicated by the percentage of mycelial growth inhibition (<10%; Lee et al. 2014). These species were evaluated for their ability to degrade PAHs. All of the studied species degraded PAHs at a higher rate than *Phanerochaete chrysosporium* KCTC6293, which is a well-known and widely used degrader of PAHs (Brodkorb Legge 1992; Figure 1). In particular, the highest degradation rates of phenanthrene (86.5%), fluoranthene (77.4%), and pyrene (82.6%) were observed with *Peniophora incarnata* KUC8836 after 2 weeks of incubation. And the effects of *P. incarnata* KUC8836 were higher, 2.1-, 1.8-, and 2.4-fold higher than *P. chrysosporium* KCTC6293 in degradation of penanthrene, fluoranthene, and pyrene, respectively. *Mycoaciella bispora* KUC8201 showed the highest degradation rate for anthracene (61.8%). And anthracene was degraded by *M. bispora* KUC8201 1.7-fold higher than the comparison species. The results indicated that the two best species, *P. incarnata* KUC8836 and *M. bispora* KUC8201, could have high endurance to toxic organic compounds and could be applied to degrade PAHs in soil environments for bioremediation. Thus, degradation of PAHs in creosote-contaminated soil was carried out by these two selected species. *Merulius tremellosus* KUC9161 was used as a comparison species for the bioremediation of soil (Lee et al. 2013).

TABLE 1 Initial Concentrations of PAHs in Creosote-Contaminated Soil

PAH		Concentration (mg kg^{-1})
1	Naphthalene	ND
2	Acenaphthylene	9.09 ± 0.75
3	Acenaphthene	39.64 ± 3.13
4	Fluorene	23.87 ± 1.80
5	Phenanthrene	44.83 ± 2.84
6	Anthracene	12.30 ± 0.86
7	Fluoranthene	27.63 ± 1.56
8	Pyrene	20.71 ± 1.14
9	Benz[*a*]anthracene	8.82 ± 0.53
10	Chrysene	11.21 ± 0.80
11	Benzo[*b*]fluoranthene	12.06 ± 1.13
12	Benzo[*k*]fluoranthene	8.75 ± 0.83
13	Benzo[*a*]pyrene	5.29 ± 0.68
14	Indeno[1,2,3-*c*,*d*]pyrene	1.24 ± 0.37
15	Dibenz[*a*,*h*]anthracene	1.42 ± 0.43
16	Benzo[*g*,*h*,*i*]perylene	2.63 ± 1.11
Σ	Total PAHs	229.49 ± 17.96

Biodegradation of PAHs in Soil

Following an incubation period of 4 weeks, comparing with the comparison species, the two selected species effectively degraded 16 PAHs in creosote-contaminated soil in this study. The initial concentration of total PAHs in soil was 229.49 mg kg^{-1} (Table 1); *P. incarnata* KUC8836 demonstrated the highest degradation rate of total PAHs in sterilized soil, to final concentration of 32.93 mg kg^{-1} (85.7%). *M. tremellosus* KUC9161 and *M. bispora* KUC8201 followed with removal rates of 75.2% and 68.7%, respectively. In addition, the abiotic control (absence of the fungus and nutrients) degraded the PAHs by 38.8% from a total initial concentration in soil due to natural attenuation (Sukor et al. 2012). The species *P. incarnata* KUC8836 showed significantly high degradation rates of fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, and benzo[*a*]pyrene, which are high-molecular-weight compounds (Figure 2). This fungal species degraded over 80% of the low-molecular-weight compounds in addition to over 60% of the high-molecular-weight compounds. The degradation of PAHs can be supported by the increase of organic matter in the soil inoculated with *P. incarnata* KUC8836 (Table 2). This soil was observed with high organic carbon content (52.34 mg kg^{-1}), resulting in strong sorption of hydrophobic intermediates such as oxygenated PAHs (Breedveld and

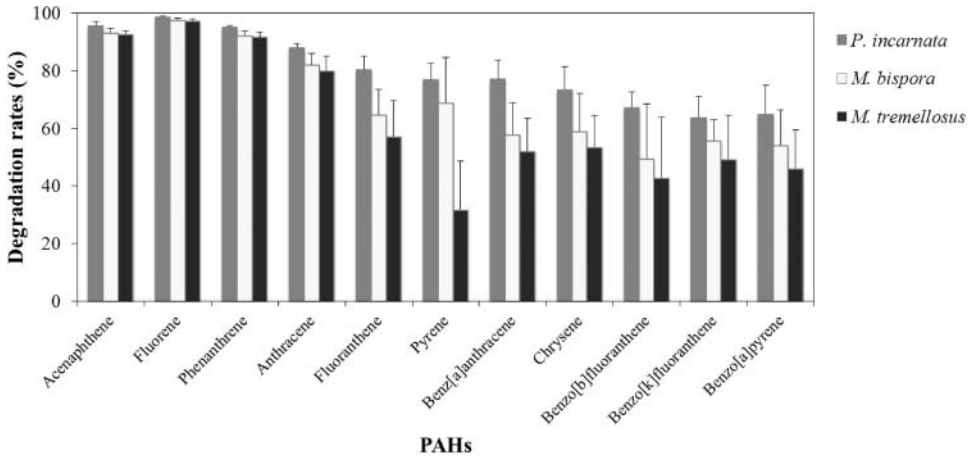

FIGURE 2 **Fungal degradation rates of PAHs in sterilized soil contaminated with creosote.**

Sparrevik 2000). Furthermore, an amount of mineralized PAHs may be correlated with the microbial activity in soil (Okere and Semple 2012). With these results, *P. incarnata* KUC8836 demonstrated the highest the activity and adaptability in soil among other species. Additionally, the contents of carbon metabolites in the fungi are stimulated by supplemental nutrient solution added to the soil to accelerate degradation. Likewise, degradation of PAHs can be enhanced by employing nutrient conditions (Lee et al. 2013). *P. incarnata* overcame anthracene removal in soil with the highest rate, despite not showing the highest efficiency of anthracene degradation in the liquid medium. Ma et al. (2013) reported that phenanthrene accelerated the pyrene degradation process, whereas anthracene, fluorene, and fluoranthene showed inhibition of pyrene degradation. In a similar way to the results, a particular PAH might inhibit anthracene degradation of *P. incarnata* KUC8836 in the liquid medium. In contrast, some of PAHs might accelerate degradation of anthracene and other PAHs as well. And the intermediates of the two- and three-ring PAHs were mineralized so that PAHs with high molecular weight can be further degraded owing to the sequential degradation (Baboshin et al. 2008). In the results, 16 PAH compounds mixed in soil might be affected by their interactions with each other's components in the degradation process. Interestingly, it was reported that *P. incarnata* was found in the highest proportion at organic acid–contaminated site, but was not reported in terms of its degradation ability of organic compounds (Sullivan et al. 2012). The results provide new information about *P. incarnata* degradative potential towards PAHs in creosote-contaminated soil. In order to better understand the efficiency of *P. incarnata* in degrading PAHs, the observation of degradation was performed by monitoring the ligninolytic enzyme activities.

Ligninolytic Enzyme Activities during Degradation of PAHs

During fungal degradation of PAHs in soil, fungi can produce ligninolytic enzymes to degrade each compound of PAHs in soil. In our study, *P. incarnata* KUC8836 showed the highest enzymatic activities of laccase and MnP among the three species (Figure 3a and b). And amount of laccase and MnP by *P. incarnata* KUC8836 was 1.7- and 1.1-fold higher than the comparison species, respectively. However, *M. tremellosus*

TABLE 2 **Physiochemical Properties of Soil**

		Extracted soil after 4 weeks incubation			
Property	Initial soil	Control soil	*P. incarnata* KUC8836	*M. bispora* KUC8201	*M. tremellosus* KUC9161
Organic matter (%)	68.48	82.88	90.24	85.03	88.98
Carbon (mg kg^{-1})	39.72	48.07	52.34	49.32	51.61
pH	5.02	5.10	5.50	5.43	5.46

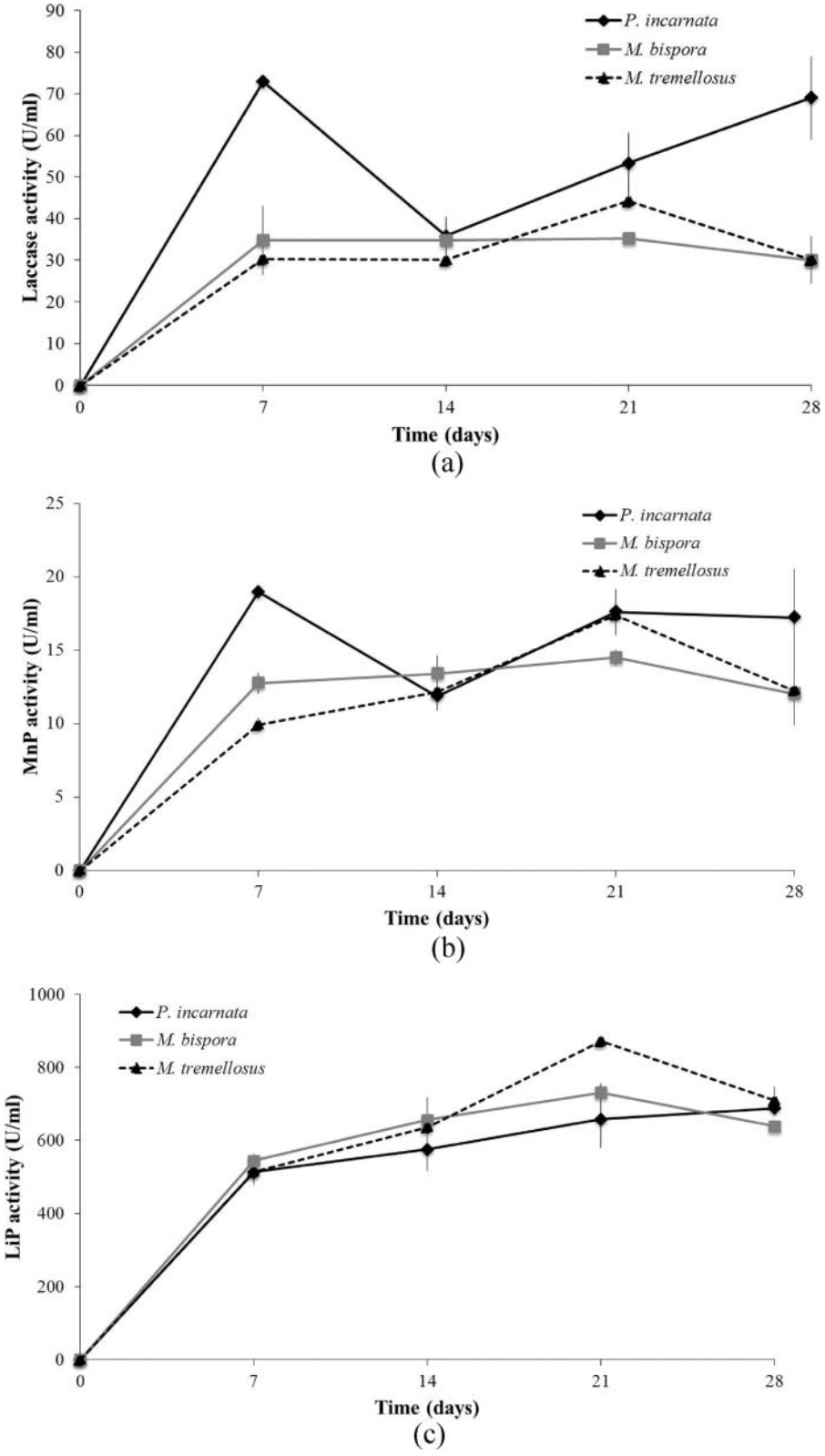

FIGURE 3 **Ligninolytic activities produced by the fungi: (*a*) laccase; (*b*) MnP; (*c*) LiP.**

KUC9161 produced LiP with the highest efficiency (Figure 3c). We postulated that the ability of *P. incarnata* to secrete elevated laccase and MnP contributed to its efficiency to degrade PAHs in soil.

Although it was reported that the efficiency of *p*-coumaric acid as a natural laccase mediator induced benzo[*a*]pyrene degradation, the mechanism of interaction between the specific enzyme and the

degradation of PAHs should be obviously defined (Giardina et al. 2010). Ligninolytic enzymes were extracellularly produced by the fungi during the mineralization of PAHs to acquire carbon sources. Laccases are oxidoreductases that directly oxidize a variety of organic compounds at the expense of molecular oxygen (Baratto et al. 2006). It was reported that any substrate with characteristics similar to not only a *p*-diphenol but also monophenols will be directly oxidized by fungal laccases (Mayer and Staples 2002). Meanwhile, MnP and LiP are peroxidases, which are initially oxidized by hydrogen peroxide, and they generate a two-electron oxidation state of the enzyme and undergo reactions in order to oxidize PAHs (Mester and Tien 2000). The results show that laccase was significantly produced in greater quantities by *P. incarnata* KUC8836 than from the other species, indicating that direct oxidation may contribute to the enhanced degradation of PAHs in soil. Each of three ligninolytic enzymes produced from both *M. bispora* KUC8201 and *M. tremellosus* KUC9161 demonstrated the highest peak on the 21st day of incubation. Meanwhile, *P. incarnata* KUC8836 produced twice as much laccase and MnP than the other species, as indicated by the highest peak, on the 7th, 21st, or 28th day of incubation, suggesting that the fungus is able to constantly mineralize the target compounds.

Moreover, *P. incarnata* KUC8836 did not actively produce ligninolytic enzymes in 2% ME liquid medium without supplemental nutrients or substrates (data not shown), demonstrating that PAHs in soil can stimulate the production of ligninolytic enzymes from the species. Although *P. incarnata* KUC8836 was previously shown to be of little significance in degrading lignin or phenolic compounds (Han et al. 2011), our findings highlighted the ability of *P. incarnata* to degrade recalcitrant organic pollutants such as PAHs efficiently. Furthermore, the species also degraded PAHs with the highest efficiency compared with other fungi that are well known as degraders of contaminants in liquid and soil environments (Lee et al. 2010, 2013). *M. bispora* KUC8201 also degraded PAHs more efficiently than the comparison species. These fungi are indigenous microorganisms found in Korea and are valuable for the bioremediation of xenobiotic compounds in soil, sediments, and other environments. And it indicates a benefit of biological inventory resources that are competitive and applicable to the local environment without risking introducing foreign fungi that might upset the natural biota in Korea.

CONCLUSION

In evaluating the degradation of PAHs by selected fungi, this study demonstrates that PAHs can be effectively removed in soil contaminated with creosote. The two species, *P. incarnata* KUC8836 and *M. bispora* KUC8201, exhibited the highest degradation efficiency due to the ability to secrete ligninolytic enzymes to catalyze recalcitrant PAHs. Of the three ligninolytic enzymes assayed, laccase might play a dominant role by directly catalyzing PAHs through direct oxidoreduction, as seen by the two peaks activities in *P. incarnata* KUC8836 during incubation. The selected white rot fungi can be useful for the bioremediation of soil, sediments, and other environments contaminated with recalcitrant organic pollutants. These valuable microorganism resources have been deposited in Korea for national competitiveness under the Kyoto Protocol Initiative.

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