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The Ability of Five Fungal Isolates from Nature to Degrade of Polyaromatic Hydrocarbons (PAHs) and Polychlorinated Biphenyls (PCBs) in Culture Media

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Abstract: The five fungal isolates (*Trametes* sp., *Polyporus* sp., and *Nigroporus* sp, fungal isolate F33 and U11) were collected from two different ecological sites in the lower northern region of Thailand. Subsequently, these five fungal isolates were tested for their ability to degrade anthracene (polyaromatic hydrocarbon) and 2,4-PCB (2,4-Polychlorinated biphenyl) in liquid culture medium. It was found that the degradation of anthracene and 2,4-PCB by *Trametes* sp., *Polyporus* sp., *Nigroporus* sp., fungal isolate F33, U11 and the control *Phanerochaete chrysosporium* IFO 311249 were all greater than 90%. The results demonstrate that the degradation of anthracene by *Trametes* sp., *Polyporus* sp., *Nigroporus* sp., fungal isolate F33 and U11 was very similar during 15 and 30 day incubations, with degradation levels ranging from 93.60 ± 1.3% to 96.40 ± 1.6%, and 96.08 ± 2.5% to 96.68 ± 1.2%, respectively. However, most of the test strains and *P. chrysosporium* IFO 311249 degraded 2,4-PCB at different rates. Of these five fungal isolates, F33 and *Polyporus* sp. exhibited the greatest ability to degrade both anthracene (96.28 ± 1.1% and 96.44 ± 2.1%, respectively) and 2,4-PCB (100%) during a 15-day of incubation.

Key words: Biodegradation, Fungi, anthracene, PAH, 2,4-PCB

INTRODUCTION

The lignin-degrading or ligninolytic enzyme systems of white rot fungi, particularly *Phanerochaete chrysosporium*, have been studied and it has been determined that lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase are three main components of these extracellular enzyme systems (Reddy, 1995; Koker *et al.,* 2000). Ligninolytic peroxidase of *P. chrysosporium* was stimulated to produce during secondary metabolism under nutrient-deficient conditions (Pointing *et al.,* 2005). Moreover, reactions of these enzymes are highly reactive and nonspecific free radicals, enabling fungi to degrade natural complex aromatic polymers of lignin as well as complex aromatic polymers that have lignin-like structure, such as pesticides, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and synthetic dyes (Cameron *et al.,* 2000; Gianfreda and Rao, 2004). Several studies have demonstrated that the ligninolytic enzyme system of *P. chrysosporium* plays an important role in catalyzing the degradation of PAHs and PCBs both in laboratory-scale studies and soil samples (Reddy, 1995; Cameron *et al.,* 2000; Yateem *et al.,* 1998; Ruiz-Aguilar *et al.,* 2002). Therefore, *P. chrysosporium* is very interesting in applications for bioremediation and biotechnology Koker *et al.,* 2000. However, biodegradation of PAHs and PCBs also occurs with other white rot fungi, such as *Bjerkandara* sp. BOS55 (Kotterman *et al.,* 1996), *Coriolus versicolor*, *Schizophyllum commune*, *Pycnoporus coccineus*, *Irpex lacteus*, *Bjerkandara adjusta*, *Coprinus cinereus, Ganoderma lucidum* (Matsubara *et al.,* 2006), *Trametes versicolor*, *Coriolopsis polyzona* (Novoty *et al.,* 1997), *Phlebia brevispora* (Kamei *et al.,* 2006) and *Pleurotus ostreatus* (Baldrian *et al.,* 2000; Kubatova *et al.,* Moeder *et al.,* 2005). In a previous report, we demonstrated that five fungal strains (*Trametes* sp., *Polyporus* sp., *Nigroporus* sp, F33 isolate and U1 isolate) had the ability to degrade 2,8-DCDD and DDT in culture media (Premjet *et al.,* 2009).

It is well known that PAHs and PCBs are mutagenic and toxic to humans and animals and are therefore potential pollutants present in soil and sedimentation. These compounds naturally degrade very slowly, so they

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remain in the environment for a long time. The objective of this study was to accelerate the degradation of PAHs and PCBs in culture media using five fungal isolates.

MATERIALS AND METHODS

***Microorganisms*:**

The five fungal strains (*Trametes* sp., *Polyporus* sp., and *Nigroporus* sp, F33 and U11) used in this study were isolated from two ecological sites in Thailand, as previously described (Premjet *et al.,* 2009). The white rot fungus, *P. chrysosporium IFO* 31249 (ATCC 34541), was purchased from the Institute for Fermentation in Osaka, Japan and used as a control for studying degradation of model compounds. Stock cultures were maintained on potato dextrose agar (PDA) slants at 4°C.

***Chemical Reagents*:**

In this experiment, anthracene [polycyclic aromatic hydrocarbons (PAHs)] and 2,4-PCB [polychlorinated biphenyls (PCBs)] were chosen as the model compounds for the biodegradation studies. The standard anthracene (Merk) and 2, 4-PCB (Riedel) were purchased from a company in Thailand.

***Culture Media*:**

I) The potato dextrose agar (PDA) was prepared by boiling 200 g of scrubbed potatoes in 500 ml of distilled water, and the liquid was strained through gauze. Then 20 g of glucose and 20 g of agar were added to the solution, followed by an additional 500 ml of distilled water. The pH was adjusted to 5.5.

II) The vitamin solution consisted of biotin 20 mg l^{-1}, folic acid 20 mg l^{-1}, thiamine hydrochloride 50 mg l^{-1}, riboflavin 50 mg l^{-1}, pyridoxal hydrochloride 100 mg l^{-1}, cyanocobalamin 1 mg l^{-1}, nicotinic acid 50 mg l^{-1}, calcium panthothenate 50 mg l^{-1}, P-aminobenzoic acid 50 mg l^{-1}, and DL-a-lipoic acid 50 mg l^{-1}.

III) The basal solution medium consisted of 10 ml of basal stock solution, $NaC_4H_4O_4.6H_2O$ 0.45 g, $C_4H_{12}N_2O_6$ 0.0221 g, glucose 2 g, and Tween-80 0.1 g, and distilled water was added to obtain a final volume of 100 ml.

IV) The basal stock solution consisted of $K_2HPO_4.6H_2O$ 2 g l^{-1}, $CaCl_2.2H_2O$ 0.5 g l^{-1}, and $MgSO_4.7H_2O$ 0.5 g l^{-1}. Mineral solution (10 ml) and vitamin solution (0.5 ml) were added, and then distilled water was added to obtain a final volume of 1 L.

V) The mineral solution consisted of nitrilotriacetic acid 1.5 g l^{-1}, $MgSO_4.7H_2O$ 3 g l^{-1}, $MnSO_4.4H_2O$ 0.713 g l^{-1}, NaCl g l^{-1}, FeSO4.$7H_2O$ 0.1 g l^{-1}, $CoSO_4.7H_2O$ 0.181 g l^{-1}, $CaCl_2.2H_2O$ 0.109 g l^{-1}, $ZnSO_4.7H_2O$ 0.178 g l^{-1}, $CaSO_4.7H_2O$ 0.01 g l^{-1}, $AlK(SO_4)_2.12H_2O$ 0.018 g l^{-1}, H_3BO_3 0.01 g l^{-1}, and $Na_2MoO_4.2H_2O$ 0.012 g l^{-1}.

All media solutions were sterilized by autoclaving prior to use.

***Culture Conditions for Biodegradation Studies*:**

The mycelia of the fungal strains that were maintained on PDA agar slants were transferred onto a PDA agar plate and incubated at 25 °C for further analysis. After 48 hr, three pieces of 5 mm (Ø) growing margin from test strains were inoculated into 10 ml of basal solution medium in a 125 ml Erlenmeyer flask with silicon stoppers. The cultures were incubated at 25 °C under static conditions for 6 days. Then anthracene and 2,4-PCB diluted in DMF (100 µl) were added to the cultures to obtain a final concentration of 0.25 mM and incubated at 25 °C for 15 and 30 days under static conditions. The medium with 0.25 mM of each compound was used to inoculate *P. chrysosporium* IFO 31249, to provide a positive control, and the negative control was medium with 0.25 mM of diluents only. Three flasks were prepared for each fungal isolate, and all culture flasks were flushed with filter-sterilized O_2 for 5 min at a flow rate of 50 ml/min every day.

***Extraction of Anthracene*:**

At the end of the 15-and 30-day incubation periods, 20 ml of acetonitrile was added to test and control cultures and solutions were shaken gently. The flasks were then sealed with a silicon stopper and sonicated in a sonication bath for 15 min and afterwards shaken by a rotary shaker at 150 rpm/min for 1 h in the dark at room temperature. The culture extraction was separated by centrifugation at 5,000 x g at 25 °C for 10 min. The supernatant was corrected and transferred to a new flask. The pellet in centrifuge bottles was washed with 20 ml of acetonitrile. This extraction was combined with the previous extraction in flask. The aqueous extraction was dried by using a Rata evaporator. The residue in flask was dissolved by 0.5 ml of hexane. This hexane extract was evaluated using GC/MS analysis.

***Extraction of 2,4-PCB*:**

Test and control cultures were extracted after 15 and 30 days of incubation. Twenty milliliters of concentrated H_2SO_4 was added to each of the controls and live cultures and blended in a stainless steel blender for 2 min. The residual 2,4-PCB was extracted with a 20 ml mixture of hexane and acetone (7:3) and centrifuged at 5,000 rpm at 25 °C for 3 min. The extraction samples were separated between solvent and aqueous phase. The extraction solvent was corrected and transferred to a new flask. The aqueous solution was re-extracted with 20 ml of mixture solvents. This extraction was combined with the previous extraction in flask. The aqueous extraction was concentrated by using a Rata evaporator until 5 ml of extraction remained. This concentrated extract was evaluated using GC/MS analysis.

***Analysis of anthracene and 2,4-PCB levels*:**

Analysis was performed using a Hewlett Packard 6890N series gas chromatograph with a 7683 series auto-sample and a 5973 series mass selective detector. Separation of anthracene and 2,4-PCB was achieved using a DB-5MS column (30 m x 0.25 mm i.d. x 25 µm). The carrier was 99.99 % helium gas at 32 cm/sec measured at 45°C in constant flow mode. The oven temperature program was 45°C for 1 min, 45-130°C at 30°C/min, 130°C for 3 min, 130-180°C at 12°C/min, 180-240°C at 7°C/min, 240-325°C at 12°C/min, and 325°C for 5 min. The injector was set to splitless mode with a 1.0 purge activation time and 300°C focus liner. The detector was set at 5973 MSD, 325°C transfer line full scan at m/z 45-450. For injection, 1 µl of each of sample was used.

The percent degradation was calculated as follows: (b/a) x 100, where a is equal to the 0.25 mM of substrate that was added to the culture medium and b is the residual substrate.

RESULTS AND DISCUSSIONS

***Degradation of Anthracene*:**

In a previous report (Premjet *et al.,* 2009), peroxisidase activity of several fungi was screened by using agar medium supplemented with Remazol Brilliant Blue R (RBBR) dye. We found five fungal isolates, *Trametes* sp., *Polyporus* sp., *Nigroporus* sp, F33 and U11 that were collected from nature and exhibited the largest decolorization zone on the agar plate. Machado *et al.,* (2005). found that only laccase activity correlated with RBBR decolorization, although some fungal strains showed increased MnP and laccase activity during RBBR decolorization, only laccase activity correlated with RBBR decolorization. Subsequently, it was determined that the RBBR decolorizing enzyme in the culture medium was laccase, which had been synthesized by *F. trogii* (Devec *et al.,* 2004) and *P. ostreatus* (Palmieri *et al.,* 2005). Additionally, it was observed that degradation of 2,8-DCDD correlated with an ability to decolorize the RBBR dye (Premjet *et al.,* 2009).

In order to evaluate the ability of the five test fungi strains to degrade another recalcitrant pollutant, all test strains were cultured in liquid culture medium with the two structurally diverse differentially chlorinated model compounds. Anthracene has no chlorine atoms attached to its benzene rings but 2,4-PCB has two chlorine atoms attached at the 2 and 4 position in the biphenyl ring of PCB. These pollutants were not degraded in flasks that had not been inoculated, demonstrating the requirement for fungal-derived enzymes.

The degradation of anthracene at day 15 of the incubation period by the five test strains ranged from 93.60 ± 1.8% to 96.60 ± 1.3%. However, the degradation percentages were slightly increased, ranging from 96.08 ± 2.5% to 96.68 ± 1.2% at day 30 of the incubation period. The degradation percentages of anthracene by the reference culture, *P. chrysosporium* IFO 31249, were 95.32 ± 1.3% and 96.72 ± 1.5%, respectively at day 15 and 30 of the incubation. There are no statistical differences ($P<0.05$) among the five fungal test strains and incubation periods. These reveal that the efficiency of the degradation of anthracene in liquid culture by the five test strains are at the same high level as *P. chrysosporium* IFO 31249, after both 15 and 30 days of incubation (Fig. 1). It was reported that LiP, which is produced by *P. chrysosporium*, has the ability to oxidize polycyclic aromatic hydrocarbons (PAHs) and anthracene with an ionization potential (IP) value of 7.55 eV and 7.43 eV, respectively (Hammel *et al.,* 1986). Subsequently, Kotterman *et al.,* (1994) observed that the activity of ligninolytic enzymes is essential for anthracene biodegradation. Moreover, degradation of anthracene was increased by addition of oxygen into the culture medium of *Bjerkandera* sp. However, Bogan and Lamar (1996) found that only the reaction of MnP, produced by *P. leavis* HHB-1625 in culture medium with low nitrogen, supported transformation of anthracene and other PAHs. In addition, it was observed that crude and purified laccase from *Trametes versicolor* and *Coriolus hirsutus* exhibited significant oxidization of anthracene and other PAHs in the presence of the redox mediator, 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (Collins *et al.,* 1996; Johannes and Majcherczyk, 2000; Cho *et al.,* 2002; Dodor *et al.,* 2004).

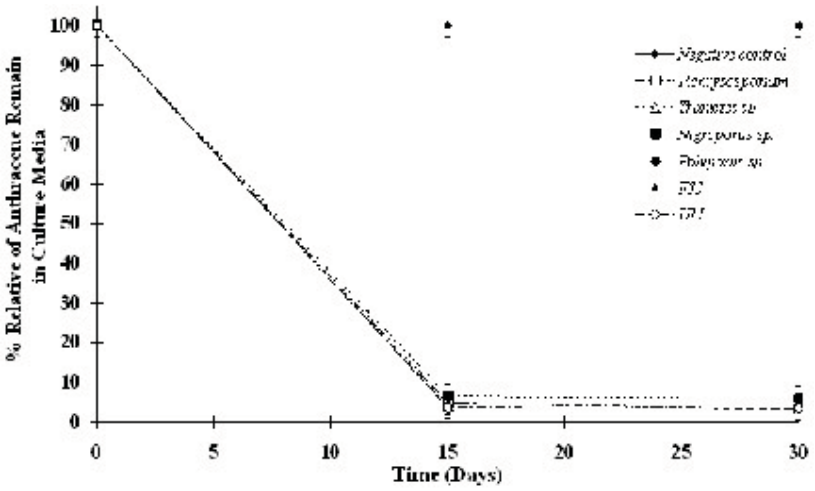

Fig. 1: Time Course of Anthracene Disappearance from Culture Media by the Five Test Strains. (Values represent the mean, n = 3, ± SE, P < 0.05)

***Degradation of 2,4-PCB*:**

After 15 days of incubation, the greatest degradation of 2,4-PCB (100%) was achieved from isolate F33, *Polyporus* sp. and *P. chrysosporium* IFO 31249, while the slightly lower degradation percentages ($P<0.05$) were observed by isolate U11 (95.60 ± 1.5 %) and *Trametes* sp. (92.20 ± 1.7%), respectively. *Nigroporus* sp. gave the lowest efficiency (62.92 ± 3.8%) of degradation of 2,4-PCB. When the incubation period was extended to 30 days, all test strains improved the efficiency of degradation of 2,4-PCB. After 30 days, complete degradation of 2,4-PCB was observed by *Trametes* sp. and isolate U11, while the efficiency of degradation by *Nigroporus* sp. was increased up to 92.80 ± 1.6%. The results indicate that the complete degradation of 2,4-PCB (100%) were obtained from the isolate F33 and *Polyporus* sp. after 15 days of incubation and *Trametes* sp. and isolate U11 after 30 days of incubation (Fig. 2). It has been reported that the number of chlorine atoms attached to the biphenyl ring greatly affects the biodegradability and mineralization of PCB mixtures by *P. chrysosporium* (Yadav *et al.,* 1995; Dietrich *et al.,* 1995). The biphenyl ring of PCB without chlorine attached at the ortho, meta or para-position is degradable by white rot fungi. Relatively high levels of degradation of aroclors 1242 and 1254 were performed both in the defined media with low and high nitrogen (Yadav *et al.,* 1995). Additionally, LiP and MnP activity of *P. chrysosporium* and some white rot fungi were found to have no direct correlation to the elimination of PCB (Krcmar and Ulrich, 1998; Beaudette *et al.,* 1998). Identification of metabolites produced during 4-4'-Dichlorobiphenyl transformation by *P. chrysosporium* were 4-chlorobenzoic acid and 4-chlorobenzyl alcohol (Dietrich *et al.,* 1995). The function of the lininolytic enzyme was not clear. Novotny *et al.,* (2004) proposed that activity of LiP, MnP and laccase produced by *Coriolopsis polyzona*, *Trametes versicolor* and *Pleurotus osteatus* were crucial for removing PCBs in liquid culture media. Kemu and Li (2004) demonstrated that hydroxyl PCBs without and with 1-6 chlorines were catalyzed by laccases of *T. versicolor* and *P. osteatus* with IP values ranging from 8.6930 (2-hydroxy) – 9.016 (4-hydroxy-PCB 26) eV. The catalyzed reaction of laccase was inhibited by the high number of chlorines in hydroxyl PCBs. Furthermore, It was observed that hydroxyl PCB with 4-6 chlorines were removed by laccase from *T. versicolor* in the presence of the mediator, 2,2,6,6-tetramethylpiperidine-*N*-oxy radical.

The biodegradability of a variety of environmentally persistent organic pollutants has been thought to result from the activity of ligninolytic enzymes produced by white rot fungi (Bogon and Larmar, 1996; Field *et al.,* 1992; Canas *et al.,* 2007). Hatakka (1994) found that some strains of white rot fungi produce lignin peroxidase (LiPs), manganese peroxidase (MnPs) and laccases, however, other strains produce only one or two of these enzymes.

Several authors have proposed that the biodegradability of organic pollutants depends on chlorine content, structure, and fungal species (Cameron *et al.,* 2000; Cameron and Aust, 1999; Field and Sierra-Alvarez, 2008). In our case, the PCB with two chlorine atoms attached at the 2 and 4 positions in the biphenyl ring was more degraded than PHA without the chlorine atom in the ring structure by fungal isolate F33 with *Polyporus* sp. during 15 days and *Trametes* sp. with U11 during 30 days. Although the enzymes that degrade PAH and 2,4-

PCB utilized by the five test strains examined in this study have not been elucidated, the degradation of these organic pollutants is assumed to take place through similar mechanisms employed by other white rot fungi. Currently, the enzymes that are responsible for degrading these recalcitrant pollutants are being investigated.

In conclusion, our results determined that all five fungal isolates showed high efficiencies in degradation of anthracene and 2,4-PCB. We also observed that the level of degradation of anthracene in liquid culture by the five fungal isolates were not statistically different ($P<0.05$) and they showed the same high level as *P. chrysosporium* IFO 31249, at both 15 and 30 days of incubation. The fungal isolate F33, *Polyporus* sp. and *P. chrysosporium* IFO 31249 showed the highest efficiency, degrading 100% of 2,4-PCB in 15 days of incubation. However, the fungal isolate U33 and *Trametes* sp. extensively degraded 2,4-PCB up to 100% within 30 days. Among the these five fungal strains, isolate F33 and *Polyporus* sp. demonstrate the greatest efficiency to degrade both anthracene and 2,4-PCB (100 %) within 15 days. Additionally, it was observed that degradation of anthracene and 2,4-PCB is highly correlated with an ability to decolorize the RBBR dye. Therefore, the RBBR decolorization assay was not only a robust screening tool to identify 2,8-DCCD but also anthracene and 2,4-PCB degrading fungi.

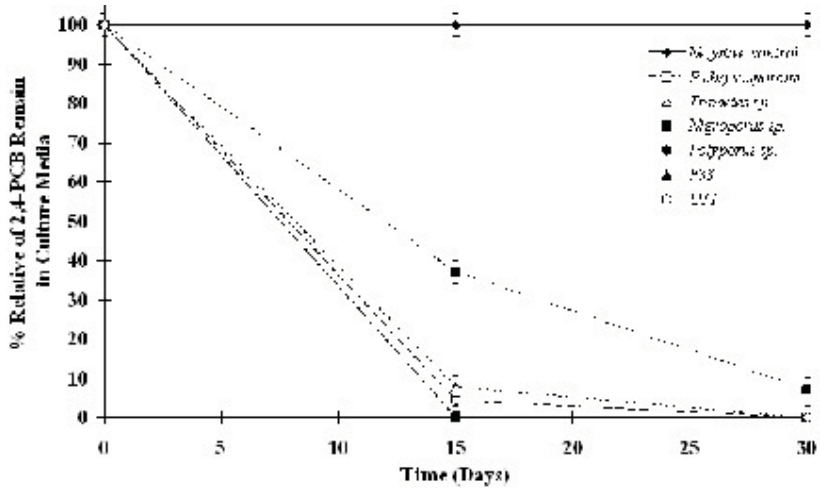

Fig. 2: Time Course of 2,4-PCB Disappearance from Culture Media by the Five Test Strains. (Values represent the mean n = 3, ± SE, P < 0.05)

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