Research article

Enhanced removal of PAHs by Peniophora incarnata and ascertainment of its novel ligninolytic enzyme genes

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Abstract

The hazardous effects of the PAHs should be managed by removal using white rot fungal ligninolytic enzymes. The white rot fungus Peniophora incarnata KUC8836 was stimulated to produce ligninolytic enzymes in a liquid medium by the addition of four substances: 0.5 g L⁻¹ Tween 80, 70 mg L⁻¹ CuSO₄·5H₂O, 10 mg L⁻¹ MnSO₄·H₂O, and 0.3 g L⁻¹ veratryl alcohol. The experiments were carried out in two different media: basal salt and 2% malt extract (ME) liquid medium. Under the experimental conditions, both laccase and manganese-dependent peroxidase (MnP) demonstrated with the highest activities in 2% ME liquid medium following the addition of Tween 80. The biodegradation of anthracene and pyrene was significantly enhanced by the induced ligninolytic enzymes when Tween 80 was added. Tween 80 is a viable co-substrate for P. incarnata, as it enhances the ability of P. incarnata to manage effective biodegradation of PAHs. Most of all, the novel laccase and MnP genes ascertained in this study, showed that the genes were involved in the production of ligninolytic enzymes from P. incarnata KUC8836.

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1. Introduction

Polycyclic aromatic hydrocarbons are of great environmental concern due to their low water solubility, toxicity, carcinogenicity and recalcitrance (Haritash and Kaushik, 2009). Anthracene and pyrene are two of the 16 PAHs defined by the US EPA as priority pollutants (ATSDR, 1995). These two PAHs exhibit representative structures of 3- and 4-fused benzene rings, respectively (Lee et al., 2014). Additionally, high molecular weight PAHs exhibit decreased solubility and increased hydrophobicity (Table 1), which increases with an increase in the number of fused benzene rings (Juhasz and Naidu, 2000). These PAHs exist as mixture in soil and persist for a long period due to their recalcitrance causing environmental problem in various sites including sediments. However, the degradation and transformation of these hazardous compounds can be efficiently treated by inoculation with white rot fungi (Husain et al., 2009).

White rot fungi have been known to be remarkable degraders of both lignin and organic pollutants, as they extracellularly produce ligninolytic enzymes to catalyze and mineralize the compounds to obtain a carbon source (Lee et al., 2010). Ligninolytic enzymes are classified as phenol oxidases and heme peroxidases (Dashtban et al., 2010). Laccase (EC 1.10.3.2), similar to multi-copper phenol oxidases, is capable of directly catalyzing the four-electron reduction of O₂←H₂O during the oxidation of phenolic compounds (Fan et al., 2011). Peroxidases, including manganese-dependent peroxidase (MnP, EC 1.11.1.13) and lignin peroxidase (LiP, EC 1.11.1.14), produced by white rot fungi oxidize the non-phenolic part of lignin in wood as well as recalcitrant xenobiotics released into the environment due to very low substrate specificities (Husain et al., 2009; Dashtban et al., 2010).

Considering the potential applications of ligninolytic enzymes in the biodegradation of xenobiotics such as PAHs, the appropriate co-substrates may be useful for the biodegradation of PAHs because they induce the secretion of catabolic enzymes and thus promote the degradation of PAHs (Hadibarata and Kristanti, 2012). As an important technical application, co-metabolism has been widely applied to the biodegradation of xenobiotics (Wen et al., 2011). Consequently, the removal efficiency can be enhanced by...
co-metabolism with induced ligninolytic enzymes from a selected fungus, Peniophora incarnata KUC8836 in order to achieve effective biodegradation of PAHs.

In our previous study, P. incarnata KUC8836 was selected and newly reported as an effective species for the degradation of PAHs among the 150 white rot fungi species (Lee et al., 2014). To enhance its degradability of anthracene and pyrene, we cultivated the fungus on media under nitrogen-limited or ordinary conditions by adding different nutrient substances to the liquid media. It is believed that the induction of enzyme production contributes to the effective degradation of PAHs due to the correlation between ligninolytic enzymes and the removal of PAHs. Thus, several inducers were chosen for the effective production of ligninolytic enzymes. Additionally, the genes encoding the novel enzyme were ascertained to comprehend the mechanisms of the ligninolytic enzymes involved in the degradation of PAHs.

2. Materials and methods

2.1. Fungal strains and liquid culture conditions

P. incarnata KUC8836 was isolated in Korea. The fungus was identified and preserved in the Korea University Culture collection (KUC). And the fungal culture was deposited in the Korean Agricultural Culture Collection (WDCM806, Suwon, Korea) as KACC53476. The fungus was maintained as a pure culture. For the induction of ligninolytic enzymes, ten fungal agar plugs (dia. 5 mm) were inoculated into a 250 ml Erlenmeyer flask containing 100 ml of liquid medium: a basal salt medium or a 2% malt extract (ME) liquid medium. The basal salt medium represents the nitrogen-limited condition, as reported by Tien and Kirk (1988): dextrose (10 g), KH₂PO₄ (2 g), MgSO₄·7H₂O (2.5 g), CaCl₂·2H₂O (0.1 g), ammonium tartrate (0.02 g), thiamine-HCl (100 mg L⁻¹), 10 ml, veratryl alcohol (4 mM; 100 ml), trace elements solution (10 ml) per 1 L of wastewater, and trace elements described by Choi et al. (2014). Optimal conditions were investigated between the two culture conditions, basal salt medium and 2% ME, and among the four supplements, 0.5 g L⁻¹ Tween 80, 70 mg L⁻¹ CuSO₄·5H₂O, 10 mg L⁻¹ MnSO₄·H₂O, and 0.3 g L⁻¹ veratryl alcohol. Each flask was then incubated at 27 °C for ten days with agitation at 150 rpm.

2.2. Assessment of ligninolytic enzyme activity

The activities of two ligninolytic enzymes, laccase and manganese-dependent peroxidase (MnP), were assayed using absorbance via a UV–Vis spectrophotometer at the wavelength 420 nm and 469 nm, respectively. After filtration of the mycelium and spores using a syringe filter (0.45 μm), the enzymatic activity of the crude supernatant was measured. To assess laccase activity, 0.1 M sodium acetate (pH 4.5) and 1.5 mM 2,2-azinobis-3-ethylbenzothiazolone-6-sulfonic acid (ABTS) were used (Lee et al., 2014). To assess MnP activity, 0.5 M sodium malonate (pH 4.5), 5 mM MnSO₄·H₂O, 1 mM 2,6-dimethoxyphenol, and 1 mM H₂O₂ were used (Lee et al., 2014). One unit of ligninolytic enzyme activity produced 1 μmol of reaction product per min at room temperature; activity was expressed in U ml⁻¹ (Lee et al., 2010). All experiments were performed in triplicate.

2.3. Degradation and analysis of PAHs

Experiments were performed using Erlenmeyer flasks (250 ml) containing 100 ml of the indicated 2% ME liquid media. A total of 0.5 g L⁻¹ Tween 80 was added to the liquid medium as a selected supplement for the induction of the ligninolytic enzymes. The fungus was aseptically inoculated into the flasks using ten agar plugs (dia. 5 mm) of fungal pre-culture grown on 2% MEA. An individual PAH was supplemented to liquid media with the addition of 50 mg L⁻¹ anthracene and 50 mg L⁻¹ pyrene dissolved in 1 ml of acetone according to the final concentration of PAHs described by Lee et al. (2013). The culture was incubated on a rotary shaker at 150 rpm (27 °C). An un inoculated abiotic control flask containing PAH was treated with the same procedures. The liquid and solid parts of the fungal culture were extracted every three days for a total of 15 days. Each sample was extracted three times with 100 ml of dichloromethane (Duskan, Korea) and was concentrated to 5 ml using a vacuum rotary evaporator (Eyela N-1000 series, Japan). The concentrations of the respective PAHs in the extracts were then determined by injecting 1 μl of the extract into a gas chromatography–mass spectrometer (GC–MS; Agilent technologies, Model 7890, Santa Clara, CA, USA). Separation was achieved using a DB-5 MS Ultra Insert column (0.25 mm diameter of 25 m length, film thickness of 0.25 μm). GC–MS analysis was conducted according to the method described by Lee et al. (2010). The corresponding ligninolytic enzymes were assessed every three days during incubation. The methods used to assess ligninolytic enzyme activity were described above.

2.4. Isolation of total RNA from Peniophora incarnata

Total RNA was isolated using an RNeasy Plant Mini kit (Qiagen, USA) and was subsequently frozen at −80 °C. First strand cDNA was synthesised from 1 μg of total RNA using the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) for RT-PCR (Real-Time-PCR) according to the provided instructions.

2.5. Amplification of the full-length cDNA of pilc1, laccase gene

Two degenerated primers, Cu1F and P-4, were used, which corresponded to the conserved amino acid sequences of the copper-binding region in fungal laccases (Table 2). PCR was performed using first strand cDNA in one cycle of incubation at 95 °C for 5 min, followed by 30 cycles of amplification at 95 °C for 1 min,
52 °C for 2 min, and 72 °C for 2 min. A final extension step at 72 °C for 5 min was then performed. To amplify the missing 5’ and 3’ ends of the fragment, two specific primers, pilc1F and pilc1R, were used for RACE-PCR (rapid amplification of cDNA ends PCR). The full-length cDNA synthesis was performed using the total RNA and a CapFishing Full-length cDNA Premix kit (Seegene, Seoul, Korea) according to the provided instructions. PCR products were electrophoresed in 1% agarose gels, purified using an AccuPrep Gel Purification kit (Bioneer, Deajeon, Korea), and cloned using the TOPO TA Cloning kit (Invitrogen). The plasmids were then extracted with an AccuPrep Plasmid Extraction kit (Bioneer, Deajeon, Korea) and sequenced (Macrogen, Seoul, Korea).

2.6. Amplification of the full-length cDNA of pimp1, MnP gene

Two degenerated primers, F2 and R2, were designed according to the metal binding regions (Table 2). PCR was performed using first strand cDNA in one cycle of incubation at 95 °C for 5 min, followed by 30 cycles of amplification at 95 °C for 1 min, 60 °C for 2 min, and 72 °C for 2 min; a final extension step at 72 °C for 5 min was then performed. The full-length cDNA synthesis of pimp1 was carried out from the MnP gene fragment with specific nested primers, pimp1F and pimp1R. The procedures used to obtain the full-length cDNA were described above.

2.7. Bioinformatic analysis of gene sequences

Analysis of the protein sequence homology among the proteins encoded by pilc1, pimp1 and other known laccase and MnP genes was carried out using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The full-length genomic DNA and the open reading frames of the cDNA of pilc1 and pimp1 were detected using Clone Manager Suite 7, version 7.04. Multiple sequence alignments of the amino acids were generated using the ClustalX 2.0.12 (Larkin et al., 2007).

2.8. Data analysis

The degradation data were analysed using the Statistical Analysis Systems (SAS 9.2, SAS Institute, Inc., Cary NC, USA) package. A one-way analysis of variance (ANOVA) followed by Tukey’s test was used to assess the significance of the different degradation values among the species. The mean values were compared using a significance level of $p < 0.05$. Degradation kinetics and rate constant were analysed and plotted using SigmaPlot Version 10.0.

3. Results

3.1. Induction of ligninolytic enzymes from P. incarnata KUC8836

To elevate the ligninolytic enzyme activities from P. incarnata KUC8836, four substances were supplemented into both the basal salt and 2% ME liquid media. Since the Kirk’s medium was reported, Phanerochaete chrysosporium has gained significant attention as a representative species for ligninolytic enzyme producers and degraders of organic pollutants throughout the world. Wang et al. (2008) reported the effects of culture conditions on the production of ligninolytic enzymes by P. chrysosporium. However, the optimal production conditions of ligninolytic enzymes from P. incarnata KUC8836 were different than those of P. chrysosporium. Laccase and MnP were highly induced from P. incarnata KUC8836 in 2% ME medium with addition of Tween 80 (Fig. 1). Since 2% ME liquid medium has been typically used in cultivation, it is easier and
faster to manufacture this medium than other media containing basal salts, resulting in 2% ME liquid medium has economic benefits (Lee et al., 2010).

Among the four substances used to enhance the production of ligninolytic enzymes, Tween 80, Mn$^{2+}$, and veratryl alcohol have been reported to induce peroxidases (Wang et al., 2008). Cu$^{2+}$ was reported as an effective inducer of phenolic oxidase (Saeki et al., 2011). In the basal medium, laccase was induced by the addition of Tween 80 and Cu$^{2+}$ compared to the control. Among the substances, there were positive effects of Tween 80 and veratryl alcohol on laccase activity, and Tween 80, Mn$^{2+}$, and veratryl alcohol had positive effects on MnP activity in 2% ME liquid medium.

3.2. Effective degradation of PAHs by P. incarnata KUC8836 and its ligninolytic enzyme activities

Two PAHs, anthracene and pyrene, were degraded within fifteen days by P. incarnata KUC8836 (Fig. 2). In our previous study, P. incarnata KUC8836 demonstrated a remarkable ability to degrade 3- and 4-ring PAHs, such as phenanthrene, fluoranthene, and pyrene (Lee et al., 2014). However, only anthracene was not effectively degraded by the fungus. Hence, Tween 80 was used to stimulate the degradation of anthracene via the induction of the ligninolytic enzymes (Fig. 3).

The removal efficiency of pyrene was enhanced by the induced ligninolytic enzymes. Laccase and MnP activities were elevated more than 2-fold when Tween 80 was added into the liquid medium compared to the unsupplemented cultures. Thus, the removal efficiency of pyrene rapidly reached 96.4% within 6 days. Meanwhile, 97.4% of the pyrene was degraded by the 15th day of incubation with P. incarnata KUC8836 alone. After the degradation of pyrene was supplemented with Tween 80, the activities of both laccase and MnP increased, which correlated with the increasing degradation of pyrene after 6 days. It is believed that pyrene degradation was greatly affected by Tween 80 and the ligninolytic enzymes.

3.3. Cloning of the full-length cDNA of the laccase and MnP genes from P. incarnata KUC8836

To detect the laccase gene from P. incarnata KUC8836, total RNA was isolated from the Tween 80-supplemented culture on the sixth day of incubation during pyrene degradation. The production of laccase was accelerated six days after the initiation of pyrene degradation. The degenerated primers Cu1F and P-4 (Table 2), which were deduced from sequenced pilc1 peptides, enabled the successful amplification of a 1167 bp product, which was identified by BLAST search as a typical laccase gene fragment. The highest homology was found with a laccase of Stropharia aeruginosa (Accession No. AFE48786). To amplify the missing sequence fragments, the specific primers Pilc1R and Pilc1F (Table 2) were derived from the 1167 bp fragment using the 5’-RACE and 3’-RACE primers, respectively. The length of the complete coding sequence

Fig. 2. Degradation rates of PAHs by P. incarnata KUC8836 supplemented with Tween 80 (a, anthracene; b, pyrene).

Fig. 3. Ligninolytic enzyme activities during degradation of PAHs (a, laccase; b, MnP).
of pilc1 was 1473 bp, which contained an intact ORF and the deduced 491 amino acids at the N-terminus (Fig. 4). And the sequence data of pilc1 was assigned with an accession number under the GenBank as KJ622360.

MnP was produced by P. incarnata KUC8836 during the degradation of anthracene. Consequently, total RNA was isolated from the Tween 80-supplemented culture on the 9th day of incubation during anthracene degradation, as the production of MnP was accelerated nine days after anthracene was degraded. From the first cDNA fragment amplified using the F2 and R2 primers (Table 2), a 294 bp fragment of a MnP gene was obtained, and this fragment was identified by BLAST search as a typical MnP gene. The highest homology was found with a MnP gene from Bondarzewia montana (Accession No. AAF33766). The 5'- and 3'- regions were extended using the RACE-PCR protocol with specific primers (pimp1F and pimp1R) and RACE primers (Table 2). The full-length cDNA clone of pimp1 (1262 bp) and its deduced amino acid sequence (358 amino acids) are indicated in Fig. 5. And the sequence data of pimp1 was assigned with an accession number of the GenBank as KJ622361.

4. Discussion

4.1. Induction of ligninolytic enzymes from P. incarnata KUC8836

P. incarnata KUC8836 is an excellent species that can be utilized for the biodegradation of PAHs and recalcitrant xenobiotics. For more rapid degradation of PAHs, the production of ligninolytic enzymes was stimulated by four substrates. Galhaup et al. (2002) reported that the transcription of the laccase gene from Trametes pubescens is induced within 10 h after the addition of 2 mM CuSO4. The results of this study showed that laccase was affected by Cu2⁺ in the basal salt medium (Fig. 1a). The presence of manganese is known to be a critical factor for the production of MnP. It was reported the maximum production of MnP occurred at a concentration of 9 mg L⁻¹ Mn2⁺ (Wang et al., 2008). The results also indicated that MnP produced in 2% ME liquid medium was induced following the addition of Mn2⁺. Veratryl alcohol could also act as a mediator for certain lignin degradation reactions through the ligninolytic catalytic formation of a veratryl alcohol cation radical (Wang et al., 2008).

Above all, Tween 80 was the greatest inducer of laccase and MnP activities for P. incarnata KUC8836. Tween 80 can increase the bioavailability of less soluble substances for the fungi and stimulate the growth of fungal spores (Hodgson et al., 2000). In the present study, Tween 80 had a significant effect on the production of laccase and MnP. Laccase activity reached 10.42 U mL⁻¹ on the 6th day in 2% ME liquid medium, which was 563% higher than the control 2% ME liquid medium culture without any supplements. MnP activity demonstrated a peak (42.18 U mL⁻¹) on the 4th day in 2% ME liquid medium, which was 1152% higher than the control 2% ME liquid medium culture. Finally, Tween 80 was selected as an effective inducer of ligninolytic enzymes in 2% ME liquid medium.
4.2. Effective degradation of PAHs by P. incarnata KUC8836 and its ligninolytic enzyme activities

The removal efficiency of anthracene was elevated following the addition of Tween 80, which enabled P. incarnata KUC8836 to produce much greater amounts of MnP. The ligninolytic enzymes have the potential to convert anthracene into dihydroxyanthracene through cis- or trans-dihydrodiol (Hadiibarata et al., 2013). In addition, pyrene was rapidly degraded six days after the addition of Tween 80. Meanwhile, Abiotic control significantly indicated a natural attenuation based on the presence of PAHs (Yu et al., 2005). Interestingly, it was increased in MnP production after 12 days of incubation during the degradation period of anthracene (Fig. 3b). Coincidentally, the degradation rate of anthracene increased after 12 days of incubation. Finally, P. incarnata KUC8836 could degrade more than 97% of the anthracene within 15 days. The results indicated that the assistance of Tween 80 may be necessary to increase the degradation rates of anthracene. The mechanism by which surfactants such as Tween 80 enhance extracellular enzyme production in filamentous fungi has not been elucidated (Wang et al., 2008). One relevant report indicated that Tween 80 transformed the cell membrane structure and promoted the release of MnP from the cell into the medium (Asther et al., 1988).

Biodegradation kinetics was determined by assuming degradation in first order with respect to the concentration of the target PAHs according to a report by LaGrega et al. (2001):

\[ \ln \left( \frac{S}{S_0} \right) = -kt \]  

where \( S \) is the remaining percentage of the individual PAH, \( S_0 \) is the initial relative percentage, \( k \) is the removal efficiency constant (day\(^{-1}\)), and \( t \) is the time (day). Plotting the natural logarithm of the relative PAH percentage with time yields a straight line of gradient \( k \) (Fig. 6). It is evident that the smallest rate constant value corresponds to that of the control, which in comparison to the other determined rate constants indicates the promotion of PAH biodegradation in the presence of the fungus and Tween 80. The \( p \) and \( F \) values for the time coefficients are lower than 0.05, implying that time is significant in the physical interpretation of the degradation of anthracene and pyrene.

The half-life of the individual PAH is determined using the following equation:

\[ t_{1/2} = \frac{\ln 2}{k} \]  

The half-life values for the fungus supplemented with Tween 80, the fungus alone, and the control were 2.6, 11.0, and 22.5 days for the degradation of anthracene, respectively. These results indicate that the addition of Tween 80 has a pronounced effect on biodegradation. The degradation of anthracene demonstrated that P. incarnata KUC8836 was stimulated 4.2-fold faster by the effect of Tween 80 than the fungus alone. Likewise, the half-life values for the fungus supplemented with Tween 80, the fungus alone, and the control are 1.3, 3.1, and 86.0 days for the degradation of pyrene, respectively. The most rapid degradation of pyrene was observed...
when Tween 80 was added to the culture. Moreover, the perceived improved biodegradation rates might be attributed to the production of ligninolytic enzymes by *P. incarnata* KUC8836, which non-specifically oxidize PAHs through the abstraction of an electron or a hydrogen atom (Lee et al., 2014; Sukor et al., 2012). Thus, the inclusion of tween 80 yielded a biodegradation rate overcoming the drawback in anthracene degradation (Lee et al., 2014). And it was reduced a time-consuming manner in effective degradation of pyrene. It is considered that Tween 80 is a valuable and cost-effective surfactant suitable for more effective degradation of PAHs.

### 4.3. Cloning of the full-length cDNA of the laccase and MnP genes from *P. incarnata* KUC8836

The full *pilc1* sequence possessed the highest homology to a laccase gene from *Meripilus giganteus* (Accession No. CBV46340), with 60% identity and 99% similarity. The multiple amino acid sequence alignments of *pilc1* and three laccase proteins, including a laccase from *M. giganteus*, were generated using ClustalX2 (Fig. 4). It was demonstrated that the *pilc1* protein contained the four copper-binding conserved domains of a typical laccase (Fan et al., 2013).

![First-order biodegradation kinetics of PAHs](image)
5. Conclusions

The hazardous effects of the PAHs should be managed by removal using white rot fungal ligninolytic enzymes. To accomplish an effective biodegradation, cost-effectiveness and time-efficiency are the most important aspects. In the present study, the ligninolytic enzymes were stimulated in 2% ME liquid medium with addition of Tween 80, which was shown as more economical than the other medium. And the tendency of increased the ligninolytic enzymes were coincident with the increased rates of PAHs-degradation. Moreover, two novel genes involved in laccase and MnP were ascertained with the full-length cDNA during the degradation of anthracene. Furthermore, *pimp1* might be useful for biodegradation and gene expression technologies at a transcriptional level. Thus, the present study described the functional analysis of a new laccase gene from *Phanerochaete chrysosporium* and a novel ligninolytic enzyme genes and showed that these genes were involved in the production of ligninolytic enzymes from *P. incarnata* KUC8836.

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