ISSN 1226-9999 (print)
ISSN 2287-7851 (online)

Korean J. Environ. Biol. 34(3) : 201~207 (2016) http://dx.doi.org/10.11626/KJEB.2016.34.3.201

Simultaneous Degradation of Polycyclic Aromatic Hydrocarbons by Attractive Ligninolytic Enzymes from *Phlebia brevispora* KUC9045

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Abstract - The hazards associated with the polycyclic aromatic hydrocarbons (PAHs) are known to be recalcitrant by their structure, but white rot fungi are capable of degrading recalcitrant organic compounds. *Phlebia brevispora* KUC9045 isolated from Korea was investigated its efficiency of degradation of four PAHs, such as phenanthrene, anthracne, fluoranthene, and pyrene. And the species secreted extracellular laccase and MnP (Manganese dependent peroxidase) during degradation. *P. brevispora* KUC9045 demonstrated effective degradation rates of phenanthrene (66.3%), anthracene (67.4%), fluoranthene (61.6%), and pyrene (63.3%), respectively. For enhancement of degradation rates of PAHs by the species, Remazol Brilliant Blue R (RBBR) was preferentially supplemented to induce ligninolytic enzymes. The biodegradation rates of the three PAHs including phenanthrene, fluoranthene, and pyrene were improved as higher concentration of Remazol Brilliant Blue R was supplemented. However, anthracene was degraded with the highest rate among four PAHs after two weeks of the incubation without RBBR addition. According to the previous study, RBBR can be clearly decolorized by *P. brevispora* KUC9045. Hence, the present study demonstrates simultaneous degradation of dye and PAHs by the white rot fungus. And it is considered that the ligninolytic enzymes are closely related with the degradation. In addition, it indicated that dye waste water might be used to induce ligninolytic enzymes for effective degradation of PAHs.

Key words : biodegradation, ligninolytic enzyme, polycyclic aromatic hydrocarbons, Remazol Brilliant Blue R, white rot fungi

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants produced from incompletely burned organic materials, oil, petroleum gas, coal, wood (Arun *et al*. 2008). PAH contamination has been received public and scientific attention owing to their recalcitrant properties and mutagenic or carcinogenic effects (Lei *et al*. 2007). Human bodies also can be damaged by PAHs via skin contact by direct inhaling and ingesting. Exposures to PAHs have been linked to skin, lung, liver, intestine, and pancreas cancers (Ting *et al*. 2011). For these reasons, many attempts have been tried to remove hazardous PAH compounds from contaminated environments. Among many process to degrade and cleavage PAHs by a variety of microbes, fungal degradation has been received great attention. One of the top abilities of white rot fungi (WRF) is to degrade lignin, a biopolymer from wood chemicals, and also a variety of hazardous chemical pollutants, such as PAHs (Suhara *et al*. 2003). WRF produce extracellular ligninolytic enzymes, laccases, manganese dependent peroxidases (MnP), and lignin peroxidases (LiP), because their very low specificity for substrate and they are able to break down the irregular structure of lignin (Casas *et al*. 2009). These enzymes are directly involved in the biodegradation of a wide range of

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xenobiotic pollutants. The ligninolytic enzymes can be induced by aromatic or phenolic compounds, such as ABTS, 2,5-xylidine, ferulic acid, or veratryl alcohol (Niladevi and Prema 2008). In addition, the use of stimulators to enhance the production of ligninolytic enzymes has been extensively approached in WRF where enzyme stimulation by aromatic compounds is well recognized (Terron *et al*. 2004; Lee *et al*. 2015).

Phlebia brevispora, a white rot fungus, has been investigated to degrade endocrine disruptors, such as polychlorinated dibenzo-*p*-dioxins/polychlorinated dibenzofurans (PCDD/PCDFs) and 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane (DDT) (Kamei *et al*. 2005; Xiao *et al*. 2011). It was also reported that chloronaphthalene, PCBs (Polychlorinated biphenyls) and PAHs were significantly mineralized by *Phlebia* species (Mori *et al*. 2003; Kamei *et al*. 2005). However, *Phlebia brevispora* is not well known to degrade recalcitrant organic pollutant.

Phenanthrene, anthracene, fluoranthene, and pyrene are listed on the standard sixteen PAHs by the U.S. EPA as priority pollutants (ATSDR 1995). And these four PAHs display representative structures of 3- and 4-fused benzene rings, respectively (Lee *et al*. 2014). PAHs are existed with mixture of PAHs and other pollutants, such as dye, heavy metal in soil, sediment environment (ATSDR 1995). Owing to its vivid biotechnological applications, ligninolytic enzyme induced by RBBR can be capable of simultaneous degradation of PAHs and decolorization of dye waste water. In this study we evaluated the degrading ability of *P. brevispora* KUC9045 against four PAHs and its ligninolytic enzymes were also quantified.

MATERIALS AND METHODS

1. Chemicals

Four PAHs used in this study, phenanthrene (PHE), anthracene (ANT), and fluoranthrene (FLT), and pyrene (PYR) with 99.0% analytical standards were purchased from the Aldrich Chemical Co., St. Louis, USA, Steinheim, Germany and China, respectively. Acetone used a solvent was purchased from Samchun Chemical Co. Ltd., Pyeongtaek-si, Korea. Remazol Brilliant Blue R (RBBR) with ~50% of dye content was purchased from Aldrich Chemical Co., Milwaukee, WI, USA. Individual PAH was initially dissolved in acetone at a concentration of 50,000 mg L^{-1}. PAHs mixture was prepared by mixing four PAHs into one stock; thus, the concentration of each PAH in this mixture was 50,000 mg L^{-1}.

2. Organisms and medium

Phlebia brevispora KUC9045 was obtained from *Picea abies* logs in Icheon, Korea and isolated on 2% malt extract agar (MEA, malt extract 20 g, agar 15 g, distilled water 1 L) amended with benomyl (4 mg) to support only the growth of basidiomycetes (Wang and Zabel 1990). To identify white rot fungi specifically, fungal DNA extraction and PCR were performed following the experimental protocols by Lim *et al*. (2005). To amplify partial large subunit (LSU) ribosomal DNA region, the PCR was conducted using the fungal universal primers, LR0R/LR3, which were used to conduct a BLAST search of the GenBank database (Altschul *et al*. 1994). DNA sequencing of PCR products were performed using the aforementioned primers and an ABI 3730XL (Macrogen, Korea). The sequences obtained in this study were deposited under the GenBank accession no. AB084614. The fungus was subcultivated every five days, and all isolates were deposited. All isolates were deposited in sterile water at 4°C in the Korea University Culture Collection (KUC), Korea University, Korea.

3. Experimental design

Experiments were performed using Erlenmeyer flasks (250 mL) containing 100 mL of 2% ME liquid medium. And they were aseptically inoculated with ten agar plugs (5 mm diameter) of fungal pre-culture. The cultures were incubated on a rotary shaker at 150 rpm (27°C) after the addition of PAHs stocks. The concentration of individual PAH was determined by 50 mg L^{-1}. Uninoculated flasks were treated similarly as an abiotic control, and *P. chrysosporium* KCTC 6293 was used as a positive control, which is well known as an effective degrader of PAHs (Bamforth and Sigleton 2005). For enhancement of degradation of PAHs, RBBR was supplemented into liquid fungal culture at concentration of 100 mg L^{-1} and 200 mg L^{-1}, respectively. With the supplement of RBBR, four PAHs stock was aseptically spiked with the concentration of 50 mg L^{-1}, respectively.

And the cultures were incubated in agitation for two weeks since fungi were inoculated in ME liquid media. All samples were carried out in triplicates.

4. Analytical methods

The entire liquid and solid part of fungal culture of *P. brevispora* KUC9045 was extracted every other day for 14 days. And the abiotic and positive control cultures were extracted on 14th day after incubation. Each sample was extracted tree times of 100 mL of dichloromethane (Duksan, Korea) to finally obtain 300 mL of each extracts. Each extracted sample was concentrated to 10 mL using a vacuum rotary evaporator (Eyela N-1000 series, Japan). The concentrations of PAHs in the extracts were measured by injecting 1 μL of the extract into a gas chromatograph-mass spectrometer (GC-MS, Agilent technologies, Model 7890, Santa Clara, CA, USA). Separation was achieved using a DB-5MS (0.25 mm diameter by 30 m length, film thickness 0.25 μm). The GC-MS analysis was conducted according to the method described by Lee *et al*. (2010).

5. Enzyme assays

For three ligninolytic enzyme activity, laccase, manganese-dependent peroxidase (MnP), Lignin peroxidase (LiP), were measured using UV-VIS spectrophotometer after removal of mycelium using a syringe filter (0.45 μm). 2% ME liquid media were used for comparing enzyme activity. The ligninolytic enzyme activity was processed according to the method described by Kirk *et al*. (1986). The LiP was assayed by measuring the oxidation of veratryl alcohol to veratraldehyde based on the increase of absorbance at 310 nm in pH 2.5 sodium tartrate buffer solution (Tien and Kirk 1988). The MnP was determined by the oxidation of 2,6-dimethoxylphenol as indicated by the increase of absorbance at 469 nm in pH 4.5 sodium malonated buffer (Wang *et al*. 2008). The laccase was measured with 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid as the substrate at 420 nm in pH 4.5 sodium acetate buffer (Novotný *et al*. 2004). One unit of enzyme activity procuced 1 μmol of reaction product per minute under the assay conditions at room temperature and the activity was expressed in U mL^{-1} (Hadibarata *et al*. 2009).

6. Data analysis

Data in terms of degradation were analyzed using the Statistical Analysis Systems (SAS 9.1, SAS Institute, Inc., Cary NC, USA). One-way analysis of variance (ANOVA) was used to assay significance of degradation among species in the tukey test. The mean values were compared by using the least significant difference index at $P < 0.05$.

RESULTS AND DISCUSSION

1. Comparison of *P. brevispora* with controls on PAHs degradation

The PAH concentrations in the cultures were determined at the end of the 14 days incubation. The percentage of degradation of phenanthrene, anthracene, fluoranthene, and pyrene in abiotic control showed 22.4%, 17.8%, 25.1%, and 14.5%, respectively (Table 1). And as a positive control, *P. chrysosporium* KCTC6293 degraded PHE (35.7%), ANT (43.1%), FLT (28.6%), and PYR (32.6%), respectively. By compared with the controls, *P. brevispora* KUC9045 demonstrated significantly higher rates of degradation of all PAHs examined in this study with PHE (66.3%), ANT (67.4%), FLT (61.6%), and PYR (63.3%), respectively ($P < 0.05$). Thus, it was concluded that the PAHs were degraded in all of the following experiments by the fungal action. In the previous study, *P. brevispora* KUC9045 showed high

Table 1. Degradation rates of four PAHs within 14 days

Species	Stran No.	Degradation rate of PAHs (%)			
		PHE	ANT	FLT	PYR
*P. brevispora**	KUC9045	66.3 (±7.8)	67.4 (±2.2)	61.6 (±11.0)	63.3 (±9.6)
P. chrysosporium	KCTC6293	35.7 (±9.9)	43.1 (±13.6)	28.6 (±12.6)	32.6 (±12.8)
Abiotic control		22.4 (±4.0)	17.8 (±1.1)	25.1 (±7.0)	14.5 (±5.3)

Numbers in parentheses are standard deviations of triplicate determinations.
The asterisk indicates means that significantly differ between the species and two controls in removal of respective PAH ($P < 0.05$).

tolerance against the four PAHs over 90%. It means the percentage of mycelial growth inhibition (MGI) of the species on MEA solid media amended with the four PAHs were expressed as $0 \leq \%MGI < 10$ (Lee *et al*. 2014). With the high resistance, *P. brevispora* KUC9045 were grown in the presence of four PAHs and the species took carbon source as a nutrient from the PAHs, mineralizing their recalcitrant structure.

2. Time course of PAHs degradation by *P. brevispora*

As time goes by, degradation rates of four PAHs by *P. brevispora* KUC9045 was increased within 14 days (Fig. 1). Among four PAHs, degradation of ANT was demonstrated with the lowest efficiency after two days of incubation. Meanwhile, at the end of 14 days incubation, *P. brevispora* KUC9045 degraded ANT with the highest rate (67.4%). It is considered that three PAHs except for ANT, were initially used as a carbon source. However, it cannot be speculated that the chemical structure of three PAHs were easy to be digested. Consequently, the species degraded ANT with the highest rate and PHE, FLT, PYR were followed by that. It indicates that probability to access three PAHs is relatively high due to more carbon presented than ANT. With the results, *P. brevispora* KUC9045 shows a valuable fungal resource for the degradation of ANT. In fact, *Peniophora incarnata*, which is known as one of the greatest degrader of PAHs, were enhanced its draw back in the degradation of ANT (Lee *et al*. 2015). Meanwhile, *P. brevispora* KUC9045 degraded ANT more than other PAHs. It indicates that there may be a major target such as ANT for the species in spite of the high tolerance against PAHs.

In addition, molar mass of three-benzen ring PAHs, which is ANT and PHE is $C_{14}H_{10}$ and four-benzene ring, which is FLT and PYR is $C_{16}H_{10}$ (Bamforth and Sigleton 2005). Because of Carbon number and molar mass might related with accessibility of fungal mycelium. In addition, the reason that densities of ANT (1.25 g m^{-3}) and PYR (1.27 g m^{-3}) were known higher than PHE (1.18 g m^{-3}) and FLT (1.25 g m^{-3}), it demonstrated slightly differ in degradation rates on 14 days.

3. Ligninolytic enzyme during degradation of PAHs

Three main ligninolytic enzymes, LiP, laccase, and MnP were measured every day during degradation of PAHs for 14 days. Among three enzymes, LiP was not produced from *P. brevispora* KUC9045. Meanwhile, laccase and MnP were produced with high efficiency during degradation of PAHs (Fig. 2).

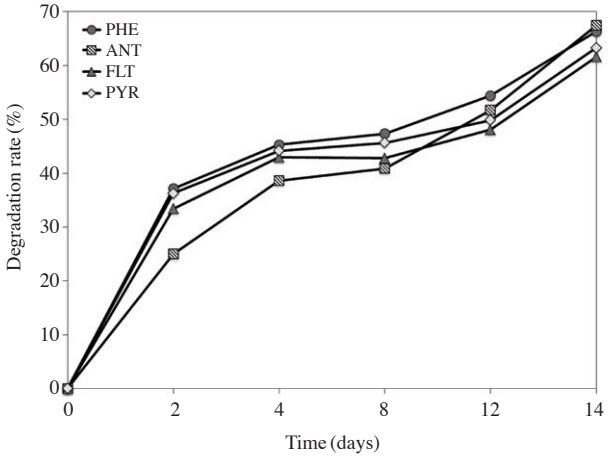

Fig. 1. Degradation rates of four PAHs by *P. brevispora* KUC9045.

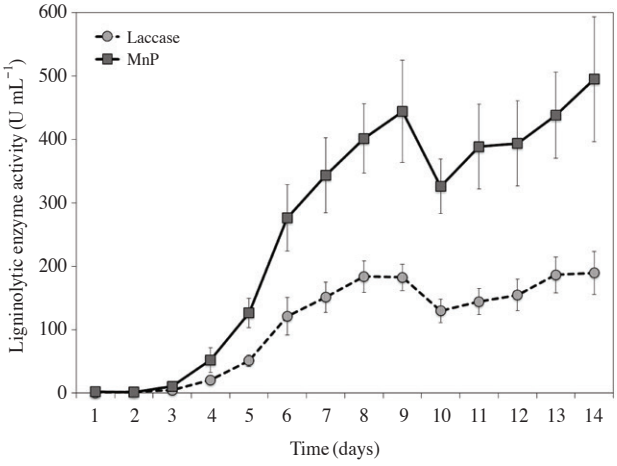

Fig. 2. Time course of producing ligninolytic enzyme during the degradation of four PAHs.

It is considered that laccase and MnP are mainly secreted and functioned during the degradation of PAHs by the species among many known enzymes. During the degradation of PAHs, there is a main enzyme for the individual fungi (Janusz *et al*. 2013). Promoter region to secrete enzyme as a protein is differentially expressed in the fungi during the mineralization of the target compound (Jang *et al*. 2012).

In time course of ligninolytic enzymes produced, trends of production of two enzymes were similarly demonstrated.

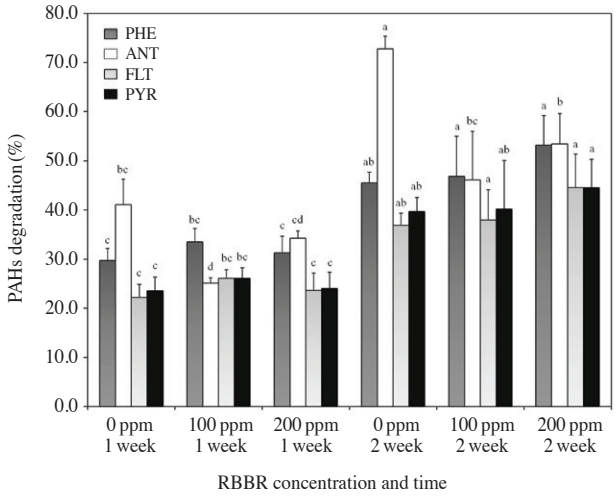

Fig. 3. Percentage of PAHs degradation after addition of RBBR. Error bars represent means ± standard deviation (n = 3). Different letters indicate statistically different means ($P < 0.05$).

On ninth day of incubation since four PAHs were spiked, laccase and MnP were produced with high efficiency and then these enzymes were temporarily decreased and eventually increased higher than on ninth day. The activity of these enzymes might be related to selectively degrade PAHs. As degradable trends of ANT were changed from 10 day of incubation, the species produced ligninolytic enzymes to focus on the target PAH. And ANT and PHE were target PAHs by *P. brevispora* KUC9045 in this study.

4. Enhanced degradation of PAHs with decolorization of RBBR

To brief the results, RBBR was supplemented into liquid media to enhance the degradation of PAHs (Fig. 3). After two weeks of the incubation, RBBR was fully decolorized (data not shown). It was photometrically monitored using 1 mL of dye content from the culture at 593 nm of UV-VIS spectrophotometer, which is the maximum wavelength for visible absorbance of this dye (Casieri *et al*., 2010). As increased decolorization rate of RBBR, four PAHs simultaneously degraded according to the different concentration of RBBR. Entirely, degradation rates of four PAHs were increased after two weeks more than one week. It indicates that PAHs can be degraded in the presence of RBBR as time goes by, and the simultaneous degradation of PAHs and dye was achieved. Interestingly, three PAHs, PHE, FLT, and PYR, were degraded in 200 ppm of RBBR more than in the media with lower concentration of RBBR. It means that the stronger and more hazardous environment force *P. brevispora* KUC9045 to degrade PAHs with higher rate. The degradation of PAHs is correlated with the production of ligninolytic enzymes. On the basic knowledge of the study, laccase and MnP were considered to play important roles in the degradation of recalcitrant organic pollutants by their induction. Laccase and MnP can be induced with supplement, and the removal of PAHs can be enhanced by the induction of ligninolytic enzymes (Lee *et al*. 2015). RBBR was used as a supplement in the study, and the degradation of the three PAHs were enhanced with decolorization of the highest concentration of RBBR. Nevertheless ANT was removed with the highest degradation rate (Fig. 1), ANT was not degraded more in the presence of the RBBR by the species. However, *P. brevispora* KUC9045 was recognized as a great degrader of ANT with the statistical degradation rate among four PAHs.

Thus, we suggest *P. brevispora* KUC9045 is valuable fungal resource for remediation of mixed contaminated enviri-onments with recalcitrant organic pollutants and dye. And it might be applicable for dedgradation of PAHs and decolorizaiton of dye waste water simultaneously.

CONCLUSION

Korean indigenous fugal isolates, *P. brevispora* KUC9045 demonstrated effective degradation of four PAHs, which is anthracne, phenanthrene, fluorancene, and pyrene, coincidentally. And the ligniniolytic enzymes could be helpful to degrade PAHs. As RBBR was supplemented, laccase and MnP were induced to produce and the degradation was enhanced by ligninolytic enzyme inducer. In addition, dye contents of RBBR were completely decolorized with the mineralization of PAHs into liquid media. In the results of our study, degradation of four PAHs could be coincidentally operated by decolorized waste water due to large amounts of ligniolytic enzymes induced by RBBR dye. Therefore simultaneous biodegradation of recalcitrant chemical pollutants and decolorization of dye waste water can be operated by indigenous fungal isolates, *P. brevispora* KUC9045. And the species is capable of biotechnological application for

degradation of a various xenobiotics.

ACKNOWLEDGEMENT

This work was was supported by the project entitled "Oil Spill Environmental Impact Assessment and Environmental Restoration (PM57431)" funded by the Ministry of Oceans and Fisheries of Korea.

REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR), 1995. Toxicological Profile for Polycyclic Aromatic Hydrocarbons (PAHs). US Department of Health and Human Services. Public Health Service, Altanta, GA.

Altschul SF, MS Boguski, W Gish and JC Wootton. 1994. Issues in searching molecular sequence databases. Nat. Genet. 6:119-129.

Arun A, RP Praveen, R Arthi, M Ananthi, KK Sathish and M Eyini. 2008. Polycyclic aromatic hydrocarbons (PAHs) biodegradation by basidiomycetes fungi, Pseudomonas isolate, and their cocultures: Comparative in vivo and in silico approach. Appl. Biochem. Biotechnol. 151:132-142.

Bamforth SM and I Sigleton. 2005. Bioremediation of polycyclic aromatic hydrocarbons; current knowledge and future directions. J. Chem. Technol. Biotechnol. 80:723-736.

Casas N, T Parella, T Vicent, G Caminal and M Sarrà. 2009. Metabolites from the biodegradation of triphenylmethane dyes by Trametes versicolor or laccase. Chemosphere 75: 1344-1349.

Casieri L, A Anastasi, V Prigione and GC Varese. 2010. Survey of ectomycorrhizal, litter-degrading, and wood-degrading Basidiomycetes for dye decolorization and ligninolytic enzyme activity. Antonie van Leeuwenhoek 98:483-504.

Hadibarata T, S Tachibana and K Itoh. 2009. Biodegradation of chrysene, an aromatic hydrocarbon by *Polyporus* sp. S133 in liquid medium. J. Hazard. Mater. 164:911-917.

Jang Y, H Lee, S Lee, YS Choi, BJ Ahn, GH Kim and JJ Kim. 2012. Cu (II)-induced molecular and physiological responses in the brown-rot basidiomycete Polyporales sp. KUC9061. J. Appl. Microbiol. 113:790-797.

Janusz G, KH Kucharzyk, A Pawlik, M Staszczak and A Paszczynski. 2013. Fungal laccase, manganese peroxidase and lignin peroxidase: Gene expression and regulation. Enzyme Microb. Technol. 52:1-12.

Kamei I, H Suhara and R Kondo. 2005. Phylogenetical approach to isolation of white-rot fungi capable of degrading polychlorinated dibezo-p-dioxin. Appl. Microbiol. Biotechnol. 69:358-366.

Kirk TK, S Croan and M Tien. 1986. Production of multiple ligninases by *Phanerochaete chrysosporium*: effect of selected growth conditions and use of a mutant strain. Enzyme Microbiol. Technol. 8:27-32.

Lee H, Y Jang, YS Choi, MJ Kim, J Lee, H Lee, JH Hong, YM Lee, GH Kim and JJ Kim. 2014. Biotechnological procedures to select white rot fungi for the degradation of PAHs. J. Microbiol. Methods 97:56-62.

Lee H, Y Jang, S Jang, YM Lee, H Lee, GH Kim and JJ Kim. 2015. Enhanced removal of PAHs by *Peniophora incarnata* and ascertainment of its novel ligninolytic enzyme genes. J. Environ. Manage. 164:10-18.

Lee H, YS Choi, MJ Kim, NH Huh, GH Kim, YW Lim, SM Kang, ST Cho and JJ Kim. 2010. Degrading ability of oligocyclic aromates by *Phanerchaete sordida* selected via screening of white rot fungi. Folia Microbiol. 55:447-453.

Lei AP, ZL Hu, YS Wong and NFY Tam. 2007. Removal of fluoranthene and pyrene by different microalgal species. Bioresour. Technol. 98:273-280.

Lim YW, JJ Kim, R Chedgy, PI Morris and C Breuil. 2005. Fungal diversity from western redcedar fences and their resistance to β-thujaplicin. Ant. van Leeuw. 87:109-117.

Mori T, S Kitano and R Kondo. 2003. Biodegradation of chloronaphthalenes and polycyclic aromatic hydrocarbons by the white-rot fungus *Phlebia lindtneri*. Appl. Microbiol. Biotechnol. 61:380-383.

Niladevi KN and P Prema. 2008. Effect of inducers and process parameters on laccase production by *Streptomyces psammoticus* and its application in dye decolourization. Bioresource Technology 99:4583-4589.

Novotný Č, K Svobodová, P Erbanová, T Cajthaml, A Kasinath, E Lang and V Šašek. 2004. Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. Soil. Biol. Biochem. 36:1545-1551.

SAS Institute Inc. SAS/STAT User's Guide, Version 9.01. Cary, NC: SAS Institute; 2004.

Suhara H, C Daikoku, H Takata, S Suzuki, Y Matsufuji, K Sakai and R Kondo. 2003. Monitoring of white-rot fungus during bioremediation of polychlorinated dioxin contaminated fly ash. Appl. Microbiol. Biotechnol. 62:601-607.

Terron MC, T Gonzalez, JM Carbajo, S Yagüe, AA Cuenca, A Tellez, ADW Dobson and AE Gonzalez. 2004. Structural closerelated aromatic compounds have different effects on laccase activity and on *lcc* gene expression in the ligninolytic fungus *Trametes* sp. I-62. Fungal. Genet. Biol. 41: 954-962.

Tien M and TK Kirk. 1988. Lignin peroxidases of Phanerochaete chrysosporium. Method Enzymol. 161:238-249.

Ting WTE, SY Yuan, SD Wu and BV Chang. 2011. Biodegradation of phenanthrene and pyrene by *Ganoderma lucidum*. Int. Biodeter. Biodegrad. 65:238-242.

Wang CJK and RA Zabel. 1990. Identification manual for fungi from utility poles in the estern United States, American Type Culture Collection, Publisher, Rockville. 356 pp.

Wang P, X Hu, S Cook, M Begonia, KS Lee and HM Hwang. 2008. Effect of culture conditions on the production of ligninolytic enzymes by white rot fungi *Phanerochaete chrysosporium* (ATCC 20696) and separation of its lignin peroxidase. World J. Microbiol. Biotechnol. 24:2205-2212.

Xiao P, T Mori, I Kamei and R Kondo. 2011. A novel metabolic pathway for biodegradation of DDT by the white rot fungi, *Phlebia lind tneri* and *Phlebia brevispora*. Biodegradation 22:859-867.

Received: 22 August 2016
Revised: 30 August 2016
Revision accepted: 30 August 2016