Biotechnol. Bioprocess Eng. 2000, 5: 211-214

Biodegradation of Pentachlorophenol by White Rot Fungi under Ligninolytic and Nonligninolytic Conditions

Won Ryul Ryu[1], Seong Hoon Shim[1], Moon Yup Jang[1], Yeong Joong Jeon[2], Kwang Keun Oh[2], and Moo Hwan Cho[1]*

[1] School of Chemical Engineering & Technology, Yeungnam University, Kyongsan, Kyongbuk 712-749, Korea
[2] Institute of Technology & Science, Cheiljedang Corporation, Ichon 467-810, Korea

Abstract The roles of lignin peroxidase, manganese peroxidase, and laccase were investigated in the biodegradation of pentachlorophenol (PCP) by several white rot fungi The disappearance of pentachlorophenol from cultures of wild type strains, *P. chrysosporium*, *Trametes sp.* and *Pleurotus sp.*, was observed. The activities of manganese peroxidase and laccase were detected in *Trametes sp.* and *Pleurotus sp.* cultures. However, the activities of ligninolytic enzymes were not detected in *P. chrysosporium* cultures. Therefore, our results showed that PCP was degraded under ligninolytic as well as nonligninolytic conditions. Indicating that lignin peroxidase, manganese peroxidase, and laccase are not essential in the biodegradation of PCP by white rot fungi.

Keywords pentachlorophenol, biodegradation, white rot fungi, ligninolytic enzymes

INTRODUCTION

Pentachlorophenol (PCP) has been used extensively as a wood preservative, pesticide, and fungicide and is rated as a priority pollutant by the EPA [1,2]. The wood preserving industry is the primary source of producing PCP containing wastewater and ground waters [3]. Several studies have reported on the biodegradation of PCP by certain bacteria, fungi and microalgae [4]. However, the process of PCP biodegradation has been observed to be very slow. Many reports showed that white rot fungi can degrade a variety of persistent aromatic organopolluntants such as PCP, PCBs, DDT, PAHs, and phenanthrene [5-12].

The enzymes of the lignin degradation system of white rot fungi have been indirectly implicated in the degradation process in a number of organic compounds including PCP [13,14]. In particular, lignin peroxidase (LiP) has been implicated in the process of degradation since partially purified ligninases preparations cleave many organic compounds [15-19]. However, the role of LiP in the degradation of many organic compounds has not been elucidated.

Baker *et al.* examined the ability of a polyphenoloxidase, the laccase of the white rot fungus *R. praticola*, to detoxify phenolic pollutants [20]. Early work by Choi showed that the ability of white rot fungi to degrade PCP [21]. And many researches showed that the role of ligninolytic enzymes in degradation of xenobiotics including PCP [22]. However Köhler found no correlation between the production of lignmases and degradation of dichlorodiphenyltrichloroethane (DDT) [16]. Dhawale found the degradation of phenanthrene by *P. chrysosporium* under ligninolytic as well as nonligninolytic conditions [17]. Konishi showed that laccase did not play an integral role in PCP mineralization [23]. Mileski *et al.* found the fungus was able to grow and mineralize PCP at concentration as high as 500 mg/L [24]

Thus, the objectives of this study were to evaluate selected white rot fungi strains, including *P. chrysosporium*, *Trametes sp.* and *Pleurotus sp.* for the biodegradation of PCP to investigate the role of lignmases in the degradation of PCP and to apply the results in the bioremediation of contaminated soils, sediments and wastewater.

MATERIALS AND METHODS

Fungal Strain

Phanerochaete chrysosporium (IFO 31249), *Trametes sp.* (KFCC 10941), and *Pleurotus sp.* (KFCC 10943) of white-rot fungi were used in this study. All the fungi were maintained on YMPG medium (yeast extract 2 g, malt extract 10 g, peptone 2 g, glucose 10 g, asparagine 1 g, KH_2PO_4 2 g, $MgSo_4 \cdot 7H_2O$ 1 g, Thiamin-HCl 1 mg) solidified with 2% agar and kept at 4°C until used.

Culture Conditions

Each disk (1 cm in diameter) from the growing edge

* Corresponding author
Tel: +82-53-810-2517 Fax: +82-53-814-8790
e-mail. mwcho@yu.ac.kr

of the mycelium on YMPG agar plates was transferred to a 300 mL Erlenmeyer flasks each containing 100 mL of a synthetic liquid medium containing 10 g/L glucose, 10 g/L malt extract, 2 g/L yeast extract, 2 g/L peptone, 1 g/L asparagine, 2 g/L KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, and 1 mg/L Thiamine·HCl in water adjusted to pH 4.5 before sterilization. And the mycelium was grown for 5 days with agitation at 200 rpm. After 5 days the mycelial mat was washed twice with sterilized water and homogenized for 30 sec in a blender, and then 0.45 mg dry cell/mL was transferred to a 300 mL flask containing 100 mL of the medium Pentachlorophenol was added to the medium after dissolved in 0.25 N NaOH solution.

The basal medium was a nitrogen-limited liquid medium consisting of 20 g glucose, 0.4 g ammonium tartrate, 2 g KH_2PO_4, 0.5 g $MgSO_4$, 0.1 g $CaCl_2$, 0.0001 g Thiamine·HCl, 0.0001 g Biotin, and 10 mL mineral stock solution (composition per liter: 1.5 g nitrilotriacetate, 3 g $MgSO_4 \cdot 7H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CuSO_4 \cdot 7H_2O$, 0 1 g $Na_2MoO_4 \cdot 2H_2O$, 10 mg $MnSO_4 \cdot H_2O$, 0.5 g $CoCl_2 \cdot 6H_2O$, 0.1 g $AlK(SO_4)_2 \cdot H_2O$, 10 mg NaCl, 0.1 g $ZnSO_4 \cdot 7H_2O$, 10 mg H_3BO_3) per liter of demineralized water. The medium was buffered in pH 4.5 with 2,2-dimethyl succinic acid (25.3 mM) And veratryl alcohol was added the medium of *P. chrysosporium*.

The medium was sterilized through filtration by using sterile membrane filters with 47 mm diameter (0.45 μm pore size). The cultures of *P. chrysosporium* were incubated at 37°C, whereas those of *Trametes sp.* and *Pleurotus sp.* were incubated at 28°C on a rotary shaker at 200 rpm. The degradation profile of pentachlorophenol was analyzed for 15 days.

Enzyme Assay

After the mycelium was removed by centrifugation, the enzyme activity was measured. Laccase activity was determined by the oxidation of ABTS. The assay mixture contained 0.5 mM ABTS, 0.1 M sodium acetate (pH 5.0), and a suitable amount of enzyme. The oxidation of ABTS was monitored by determining the increase in A_{420} (ε_{420}, 3.6×10^4 $M^{-1} \cdot cm^{-1}$).

Lignin peroxidase (LiP) activity was estimated by monitoring the oxidation of veratryl alcohol to veratryl aldehyde as indicated by the increase in A_{310} (ε_{310}, 9.3×10^3 $M^{-1} \cdot cm^{-1}$). The assay mixture contained 25 mM sodium tartarate (pH 2 5), 2 mM veratryl alcohol, 0.4 mM H_2O_2, and 50 to 275 μL of extracellular fluid with a total volume of 0.5 mL. The reaction was initiated by adding H_2O_2.

Manganese peroxidase (MnP) activity was estimated by monitoring the oxidation of phenol red spectrophotometrically at 610nm (ε_{610}, 1.21×10^4 $M^{-1} \cdot cm^{-1}$). The reaction mixture contained 50 mM sodium succinate (pH 4.5), 50 mM sodium lactate (pH 4.5), 0 1 mM $MnSO_4$, 3 mg of gelatin per mL, 50 μM H_2O_2, and 0.1 mM phenol red. The reaction was initiated by adding H_2O_2 and was conducted at 30°C. The mixture was sampled by removing 1 mL out of the total 5 mL volume and adding it to 40 μL of 5 N sodium hydroxide every minute for 4 min One unit of enzyme activity was equivalent to 1 μmol of the product formed per min.

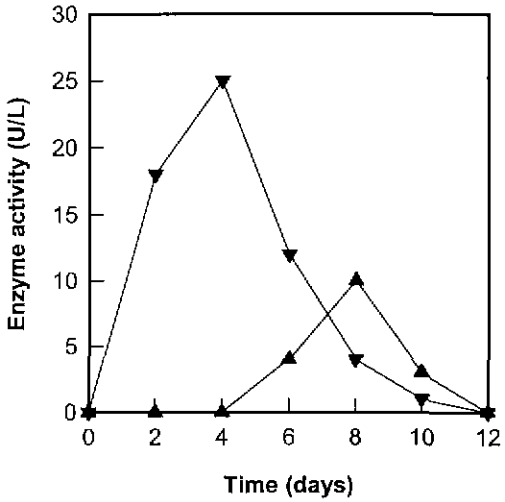

Fig. 1. Enzyme activity in *P. chrysosporium* cultures. Symbols: ▼, MnP activity (U/L); ▲, LiP activity (U/L).

Gas Chromatography

The concentration of PCP in the sample was determined by gas chromatography; PCP was extracted with hexane. 0.5 μL extract was analyzed on a CBP5 fused silica capillary column (25 m × 0.22mm × 0.25 μm; Shimadzu) installed in a Shimadzu GC-14A gas chromatography equipped with FID. Split injection (1:100) and programmable temperature were used with nitrogen as the carrier gas The oven temperature was 80°C for 1 min, increased to 180°C at a rate of 20°C/min, increased to 220°C at a rate of 4°C/min, increased to 280°C at a rate of 20°C/min, and maintained at 280°C for 5 min. The injection temperature was 250°C and the detector temperature was 250°C. Standards were run and used to figure out PCP concentration To determine the PCP concentration in the biomass, the mycelium was suspended in 0 25 N NaOH, homogenized in a blender and then centrifuged. The supernatant was used for analysis using the above described method.

RESULTS AND DISCUSSION

White rot fungi, grown in the form of mycelial pellets for 15 days, were used to examine the role of extracellular enzymes and removal of PCP. Since many previous studies have used pH of 4.1 or above, further experiments using white rot fungi were performed using media with pH 4.5 Fig. 1 shows ligninases activity of *P. chrysosporium* during cultivation without PCP addition. As shown in Fig. 1, the maximum MnP activity was 25 U/L on the 4th day of incubation and the

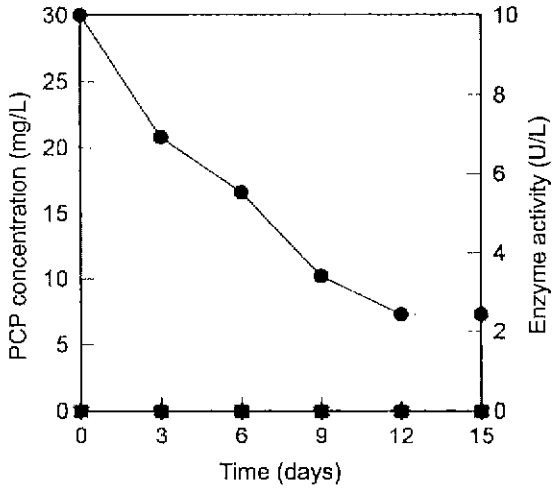

Fig. 2 Biodegradation of PCP in *P. chrysosporium* cultures Symbols: ●, PCP concentration (mg/L), ▼, MnP activity (U/L); ▲, LiP activity (U/L), ■, laccase activity (U/L).

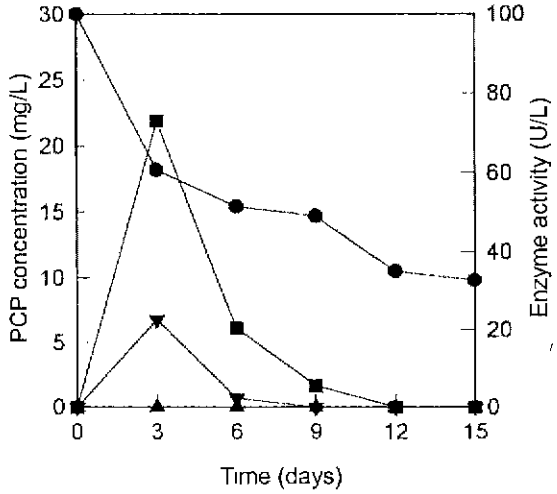

Fig. 3. Biodegradation of PCP in *Trametes sp.* cultures. Symbols: ●, PCP concentration (mg/L); ▼, MnP activity (U/L); ▲, LiP activity (U/L), ■, laccase activity (U/L)

maximum LiP activity was 10 U/L on the 8th day of incubation. Ligninases activity was extremely low.

Fig. 2 shows the biodegradation of PCP by *P. chrysosporium* under nonligninolytic condition. During the cultivation of *P. chrysosporium*, PCP was rapidly degraded within 15 days of incubation (Fig. 2). After 15 day culture period, approximately 76.0% of 30 mg PCP/L was degraded. However, lignin peroxidase (LiP) and manganese peroxidase (MnP) activities were not detected in the culture broth of *P. chrysosporium* containing PCP during 15 days (Fig. 1). Comparison of Fig. 1, these results presumed that inhibition on production of enzyme by PCP These data indicated that PCP was degraded under nonligninolytic conditions.

Fig. 3 shows the biodegradation of PCP by *Trametes sp* Laccase and MnP activities in the culture broth of

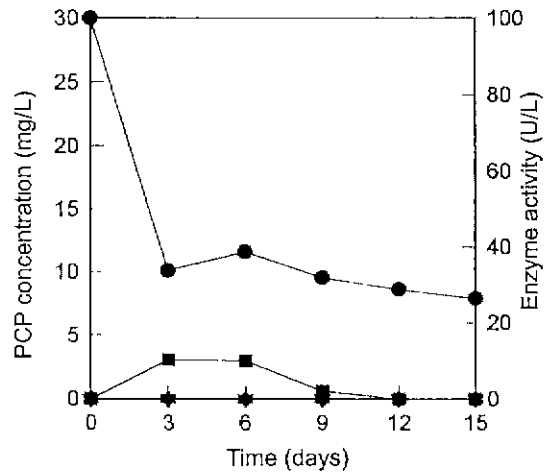

Fig. 4. Biodegradation of PCP in *Pleurotus sp* cultures. Symbols ●, PCP concentration (mg/L), ▼, MnP activity (U/L); ▲, LiP activity (U/L); ■, laccase activity (U/L)

Table 1. Concentrations of PCP after 15 days of culturing

Strain	Time[a]	Remained PCP concentrations (mg/L)		
		Extracellular	Mycelia	Total
P. chrysosporium	15 days	7.2	1.0	8.2
Trametes sp.	15 days	9.8	1.5	11.3
Pleurotus sp.	15 days	7.9	1.4	8.9

[a] Incubation time after the addition of 30 mg PCP/L.

Trametes sp. were detected The maximum laccase activity was 88.3 U/L on the 3rd day of incubation at which 39.3% of the initial amount of PCP was degraded. The degradation of PCP reached to the maximum level of 67.3% after 15 days of incubation. These data indicated that PCP was degraded under ligninolytic conditions.

Fig. 4 shows the degradation of PCP by *Pleurotus sp.* During 15 days of cultivation, approximately 73.7% of initial PCP was degraded Laccase activity in the culture broth of *Pleurotus sp.* was determined With respect to the degradation of PCP, *P. chrysosporium* was the most efficient degrader among the strains we examined.

Table 1 shows the adsorption of PCP by biomass. After 15 days, the amount of PCP in the liquid phase and the biomass phase was determined. The data listed in Table 1 show that most of the PCP in the feed solution was degraded by the active fungi and not simply taken up by the mycelia. These results clearly suggested that the mycelia were able to degrade the initially adsorbed PCP. PCP is firstly adsorbed on cells, PCP is slowly desorbed from cells and is degraded by enzymes.

Since lignin-degrading enzymes are produced under nitrogen limiting condition [18], they have been indirectly implicated in the degradation of a wide variety of organic compounds including PCP [5,6]. Studies have also shown that purified ligninases can catalyze the oxidation of various organic compounds [19]. Thus, the

lignin degradation system of the fungi has been directly as well as indirectly linked to the degradation of various compound remains including PCP. Despite many researches, the role of ligninases in the degradation of various compounds remains unclear. According to Köhler *et al.* [16], extracellular ligninases play no role in the degradation of DDT. And, according to Dhawale *et al.* [17], phenanthrene was degraded under ligninolytic as well as nonligninolytic conditions.

In view of these conflicting reports, we felt that a genetic approach would help to clarify the roles of LiP, MnP, and laccase in the process of degradation. Our results showed that the lignimolytic enzyme activity of *Trametes sp.* in culture broth was higher than those of *P. chrysosporium* and *Pleurotus sp.* The lignimolytic enzyme activity was not determined in the culture broth of *P. chrysosporium*. However, the degradation of PCP by *P. chrysosporium* was the highest among the strains that we examined. Our results showed that PCP was degraded under lignimolytic as well as nonligninolytic conditions. Therefore, the results collectively indicate that the ligninolytic enzymes are not essential for the degradation of PCP by white rot fungi. It is estimated that other enzymes which are more important than ligninolytic enzymes are concerned in degradation of PCP.

Acknowledgements The authors acknowledge the financial support from the Ministry of Commerce, Industry and Energy, Republic of Korea.

REFERENCES

[1] Lamar, R. T., M. J. Larsen, and T. K. Kirk (1991) Sensitivity to and degradation of pentachlorophenol by *Phanerochaete* spp.. *Appl. Environ. Microbiol.* 57: 2085-2090.

[2] Lasen, S., H. V. Hendriksen, B. K. Ahring (1991) Potential for thermophilic (50°C) anaerobic dechlorination of pentachlorophenol in different ecosystems. *Appl. Environ. Microbiol.* 57. 2085-2090.

[3] Crosby, D. G., K. I. Benyon, P. A. Greve, F. Korte, G. G. Still, and J. W. Vouk (1981) Environmental chemistry of pentachlorophenol. *Pure Appl. Chem.* 53: 1051-1080.

[4] Okeke, B. C., A. Paterson, J. E. Smith, and I. A. Watson-Craik (1997) Aerobic metabolism of pentachlorophenol by spent sawdust culture of "shiitake" mushroom in soil. *Biotechnol. Lett.* 15: 1077-1080, *Appl. Microbio. Biotechnol* 48: 563-569.

[5] Bumpus, J. A., M. Tien, D. Wright, and S. D. Aust (1985) Oxidation of persistent envrontal pollutants by a white rot fungus. *Sci.* 228: 1434-1436.

[6] Mileski, G. J., J. A. Bumpus, S. D. Jurek, and S. D. Aust (1988) Biodegradation. *Appl. Environ. Microbiol.* 54· 2885-2889.

[7] Bezarel, L., Y. Hadar, and C. E. Cerniglia (1996) Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 62: 292-295.

[8] Bumpus, J. A. and B. Brock (1988) Biodegradation of crystal violet by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 58: 3598-3604.

[9] Cerniglia, C. E., P. P. Fu, and S. K. Yang (1982) Metabolism of 7-methyl-benzo[a]anthracene and 7-hydroxymethylbenz[a]anthracene by *Cunninghamella alegance*. *Appl. Environ. Microbiol.* 44: 682-689.

[10] Field, J., E. de Jong, G. Feijoo-Costa, and J. de Bont (1993) Screening for lignimolytic fungi applicable to the biodegradation of xenobiotics. *Trends Biotechnol.* 11: 44-49.

[11] Hammel, K. E., B. Kalyanaraman, and T. K. Kirk (1986) Oxidation of polycyclic aromatic hydrocarbon and dibenzo[p]dioxins by *Phanerochaete chrysosporium* lignmase. *J. Biol. Chem.* 261: 16948-16951.

[12] Spadaro, J., M. Gold, and V. Renganathan (1992) Degradation of dyes by the lignin-degrading fugus *Phanerochaete chrysosporium*. *Appl. Microbiol. Biotechnol* 58: 3598-3604.

[13] Fenando, T., J. A. Bunpus, M. Tien, D. Wright, and S. D. Aust (1990) Biodegradation of TNT by *Phanerochaete chrysosporium*. *Appl. Envion. Microbiol.* 56: 1666-1671.

[14] Kennedy, D. W., S. D. Aust, and J. A. Bunpus (1990) Comparative biodegradation of alkyl halide insecticides by the white rot fungus, *Phanerochaete chrysosporium* (BKM-F-1767). *Appl. Envion. Microbiol.* 56: 2347-2353.

[15] Ceriglia, C. E. and S. K. Yang (1984) Stereoselective metabolism of anthracene and phenanthrene by the fungus *Cunninghamella elegans*. *Appl. Envion. Microbiol.* 47: 119-124.

[16] Köhler, A., A. Jäger, H. Willershausen, and H. Graf (1988) Extracellular ligninase of *Phanerochaete chrysosporium* Burdsall has no role in the degradation of DDT. *Appl. Envion. Microbiol* 29: 618-620.

[17] Dhawale, S. W., S. S. Dhawale, and D. Dean-Ross (1992) Degradation of Phenanthrene by *Phanerochaete chrysosporium* occurs under lignimolytic as well as nonlignimolytic conditions. *Appl. Envion. Microbiol.* 58: 3000-3006.

[18] Bumpus, J. A. and S. D. Aust (1989) Biodegadation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. *Appl. Envion. Microbiol.* 55: 154-158.

[19] Aust, S. D. (1990) Degradation of environmental pollutants by *Phanerochaete chrysosporium*. *Microbiol. Ecol.* 20: 197-209.

[20] Baker, W. L., K. Sabapathy, M. Vibat, and G. Lonergan (1996) Laccase Catalyzes Formation of an Indamine Dye between 3-methyl-2-benzothiazolinone hydrazone and 3-dimethylaminobenzoic acid. *Enzyme and Microbial Tech.* 18: 90-94.

[21] Choi, S. H., E. Song, M. B. Gu, and S. H. Moon (1998) A study on synthesis of lignin peroxidase and degradation of pentachlorophenol (PCP) by *Phanerochaete chrysosporium*. *Kor. J. Biotechnol. Bioeng.* 13: 223-230.

[22] Sangard, D., M. S. A. Leisola, and A. Fiechter (1986) Role of extracellular ligninases in biodegradation of benzo[a]pyrene by *Phanerochaete chrysosporium*. *Enzyme Micro. Technol.* 8: 209-212.

[23] Konishi, K. and Y. Inoue (1972) Detoxification mechanism of pentachlorophenol by laccase of Coriolus versicolor. *Mokuzai Gakkaishi* 18: 463-469.

[24] G. J. Mileski, J. A. Bumpus, M. A. Jurek, and S. D. Aust (1988) Biodegration of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Microbiol. Biotechnol* 54· 2885-2889.

[Received March 17, 2000, accepted June 19, 2000]