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Degradation of Pentachlorophenol by Lignin Degrading Fungi and Their Laccases*1

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ABSTRACT

The degradation of pentachlorophenol (PCP) by lignin degrading fungi was performed. Several fungi, *Abortiporus biennis*, *Cerrena unicolor* and *Trametes versicolor*, were tested to evaluate the inhibitory effect of PCP on their growth. At the extremal concentration of PCP (500 μM), only *C. unicolor* showed relatively fast growth (60% within 14 days) in the comparison to the control culture. In the case of *A. biennis* and *C. unicolor*, when initial PCP concentration was 50 μM, about 88.2% and 79.5% of PCP degradation were achieved within 3 days, respectively. When 2,5-xylidine (0.2 mM) was added to the *C. unicolor* culture, as high as 98% of PCP degradation was achieved within just an hour after its addition. *A. biennis* removed 44% of PCP at the same condition. PCP was completely disappeared when laccase activities reached to maximum.

Keywords : laccase, lignin degrading fungi, *Abortiporus biennis*, *Cerrena unicolor*, *Trametes versicolor*, pentachlorophenol (PCP), 2,5-Xylidine

1. INTRODUCTION

Recently efforts have been made toward improving biodegradation of toxic compounds in the environment. Especially contamination by chlorinated aromatic compounds has been the subject of research. As conventional (chemical) methods of wastewater treatment such as activated sludge or aerated lagoons are rather ineffective (Bergbauer *et al.*, 1991), recently biological methods received more attention. Among organisms showing biodegradation potency, wood rotting terrestrial fungi seem to be the most promising. These fungi, which are capable to degrade lignin present in wood show also potency to degrade many structurally diverse organic pollutants (Kang and Stevens, 1994). Accumulation of toxic aromatic chemicals causes meaningful environmental problems. For example some polycyclic aromatic hydrocarbons or chlorinated phenols are recalcitrant in the environment, often carcinogenic, mutagenic or toxic

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and pose a serious risk to human health (Blumer, 1976; Haggblom and Young, 1990, Pellinen *et al.*, 1988). Especially chlorophenols are common environmental contaminants because they have been extensively used as biocides, mainly as the wood preservatives (Haggblom and Young, 1990). Chlorinated phenols and other chlorinated aromatic compounds are also formed as byproducts when chlorine is used for bleaching of pulp and disinfection of drinking water and waste water containing phenols (Rao, 1978).

Pulp mill effluents from bleaching procedures are dark brown due to the presence of chromophoric, polymeric and chlorinated lignin derivatives. These chlorinated compounds possessing toxic and mutagenic properties refused into streams and oceans may accumulate in aquatic organisms (river or sea plants and fishes) resulting in serious environmental and health problems (Pellinen *et al.*, 1988). Low molecular weight components of the first alkali extraction stage effluent (E1 effluent) of a kraft bleach mill comprise various chlorinated organics, of which the chlorinated phenols are a major component. The quantity of chlorinated phenols is not too high in comparison with the total quantity of organic substances in the E1 effluent. However, these chlorinated phenols are generally considered as being responsible for a major part of the toxicity of the E1 effluent (Pellinen *et al.*, 1988). The pentachlorophenol (PCP) is a commonly occurring organopollutant that has long been the subject of environmental research (Badkoubi *et al.*, 1996). A number of approaches for degradation or transformation of PCP in soil and ground water have been reported (Konishi and Inoue, 1972; Badkoubi *et al.*, 1996; Cho *et al.*, 2001). The promising results using lignin degrading fungi to mineralization PCP in soil and ground water were reported by Lamar *et al.* (1990), and Kang and Stevens (1994), respectively.

It is generally believed that laccase enzyme is produced by several basidiomycete, ascomycete and deuteromycete fungi. This enzyme is induced by 2,5 xylidine which has been firstly proposed by Fahraeus *et al.* (1958) in the case of *Trametes versicolor*. They found over 160-fold stimulation of laccase when this compound was introduced into the growing culture. Afterwards it was confirmed that 2,5-xylidine among tested 17 compounds showed the highest inducibility of laccase from tested white-rot fungi (Leonowicz *et al.*, 1997; Cho *et al.*, 1998).

In this study the effect of lignin degrading fungi, *Abortiporus biennis, Cerrena unicolor* and *Trametes versicolor*, and 2.5-xylidine induced extracellular laccase on the degradation of chlorinated pollutants PCP were studied.

2. MATERIALS and METHODS

2.1. Fungi

All fungal strains, *Abortiporus biennis* (Bull.ex Fr.) Sing. (MC T060), *Cerrena unicolor* (Bull.ex Fr.) Murr. (MC T143) and *Trametes versicolor* (L. ex Fr.) Pil. (FPD 4838), were obtained from the Culture Collection of the Department of Wood and Paper Science, Chungbuk National University, Cheongju, Republic of Korea. The fungal cultures were maintained on 2% (wt/vol) malt agar slants.

2.2. Determination of Laccase Activity

Laccase activity of the culture fluid and of the purified free and immobilized enzymes (Leonowicz *et al.*, 1997; Cho *et al.*, 1998; Ginalska *et al.*, 2001a; 2001b; Kim *et al.*, 2002) was measured at 20°C and pH 5.6 in 0.1 M McIlvaine (1921) citrate phosphate buffer according to Bollag and Leonowicz (1984) on a

Shimadzu Graphic Printer PR1 spectrophotometer with syringaldazine as a substrate (Leonowicz and Grzywnowicz, 1981). To exclude endogenous peroxide, the 10 min. preincubation (stirring) of the enzyme sample with catalase (10 mg/㎖) was performed. The laccase activities were expressed as ncat, *i.e.* nmoles of substrate oxidized during one second, by assuming a molar absorption coefficient of 65,000 (Leonowicz and Grzywnowicz, 1981; Leonowicz *et al.*, 1999) for the colored (red) reaction product. The laccase activities were measured using the reaction mixtures containing the enzyme in 0.1 M citrate phosphate buffer with pH optima for particular fungi.

2.3. Degradation of Pentachlorophenol (PCP)

For Petri dish experiment, SMY (sucrose-malt extract-yeast)-agar medium contained 10 g sucrose, 10 g malt extract, 4 g yeast extract and 20 g agar in 1000 ㎖ deionized water. Fungal strains were inoculated onto 20 ㎖ of SMY-agar medium containing 50, 100, 250 and 500 μM of PCP in a 90 mm Petri dishes and cultivated at 27°C. Fungal growth was measured every day as colony diameter in mm.

For Erlenmayer flask experiment, pieces of mycelia (*ca.* 0.5 cm) were transferred from the Petri dishes cultures grown on the SMY-agar media into the sterile (0.1 MP, 45 min.) liquid Lindeberg and Holm (1952) media (20 ㎖ in 100 ㎖ Erlenmayer flasks). Before sterilization, the pH's were adjusted by 1 M HCl to optimal values for each strain (5.0 for *A. biennis* and *T. versicolor,* and 4.5 for *C. unicolor*). The cultures were grown at 25°C till the mycelia made confluent mat, and then PCP was added to concentrations 50 μM and 100 μM, respectively.

For studying the influence of 2,5-xylidine as an inducer of laccase during PCP degradation, it (0.2 mM) was added at 3rd day after inoculation, and the cultures were continuously grown at 25°C. All experiments were performed in triplicate.

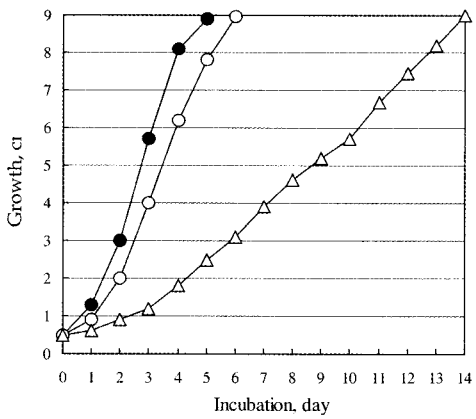
2.4. HPLC Analysis of PCP

The concentrations of PCP remaining in cultures were determined by HPLC (high-performance liquid chromatography) using a Hewlett Packard HPLC 1100 series equipped with DAD (diode array) detector and Hewlett Packard ODS-Hypersil column (200 mm × 4.6 mm, particle size 5 μm). PCP was extracted from 5 ㎖ sample of cultures with the addition of 5 ㎖ n-hexane, mixing, centrifugation (3 min at 5,000 rpm), and filtering the supernatant through 0.45 μm membrane filters (Advantec Inc. USA). The pellet was once more extracted with the same amount of n-hexane and centrifugated. The supernanants were collected, concentrated completely in vacuum at room temperature and finally diluted in 5 ㎖ ethanol (HPLC grade) for injection into HPLC system, where the mobile phase consisted of acetonitrile and 0.05% phosphoric acid (8:2 for PCP, vol/vol). The flow rate of the mobile phase was 1 ㎖/min. The concentrations of chlorophenols were measured at absorbence 218 nm for PCP, and calculated with using a Hewlett Packard Chemstation.

3. RESULTS and DISCUSSION

3.1. Effect of PCP on Fungal Growth

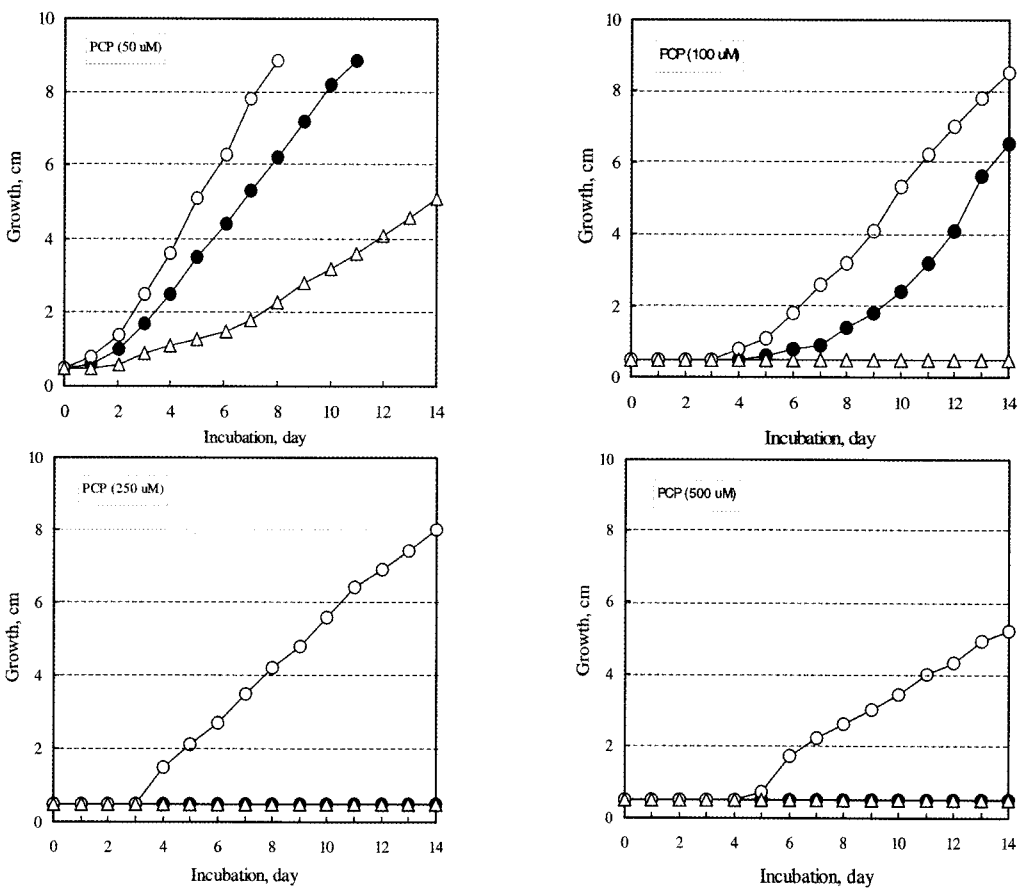
The growth rates of tested fungi on 2% agar media are presented in Fig. 1. *A. biennis* and *C. unicolor* reached to maximum growth within 5~6 days, whereas, *T. versicolor* 98.5% of growth within 14 days. Fig. 2 showed the effect of different PCP concentration on fungal growths. When fungal cultures were inoculated into the



• *A. biennis* ○ *C. unicolor*, △ *T. versicolor*

Fig. 1. The mycelial growth of fungi at the control media.

agar medium containing 50 μM PCP, *C. unicolor* showed relatively fast growth, but those of *A. biennis* and *T. versicolor* were slower. Especially, *T. versicolor* was grown slowly during 14 days only about 60% in comparison with the control culture without PCP. At 100 μM of PCP concentration, *C. unicolor* also showed still good growth compared to the other fungi. At the 250 and 500 μM of PCP concentrations, *A. biennis* and *T. versicolor* were totally inhibited. On the other hand, *C. unicolor* showed the highest tolerance to PCP, and even at 500 μM, its growth was enough to reach more than the half agar zone within 14 days.



• *A. biennis* ○ *C. unicolor* △ *T. versicolor*

Fig. 2. Effect of different PCP concentration on fungal growth.

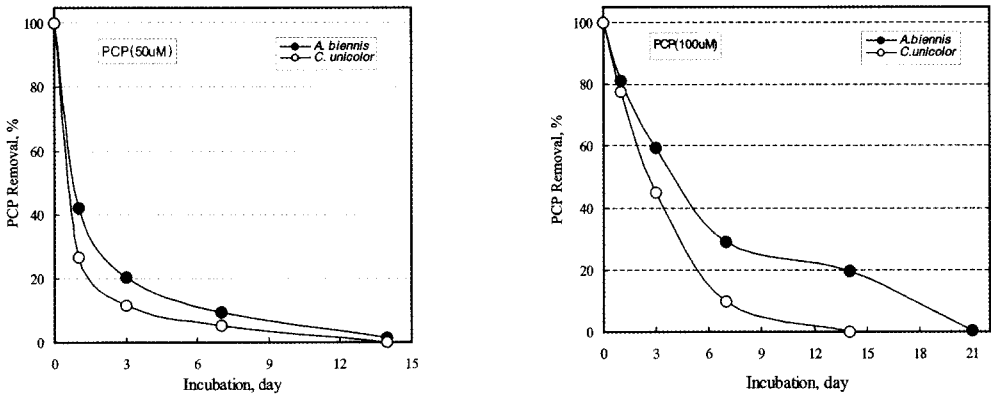


Fig. 3. Biodegradability of PCP by *A. biennis* and *C. unicolor*.

According to these results, *C. unicolor* showed the highest tolerance on PCP compared to the other fungi. Lamar *et al.* (1990) reported that lignin degrading fungus, *Phanerochaete chrysosporium,* can be used for biodegradation of PCP at 250 to 400 μM concentration. Among the heterogeneous group of white rot fungi, *P. chrysosporium* is usually considered as the most suitable fungus for efficient degradation of the recalcitrant chlorinated compounds (Bergbauer *et al.*, 1991). In this study *C. unicolor* can be considered as one of the most tolerant fungi to the recalcitrants. Bergbauer *et al.* (1991) reported that chlorinated lignin derivatives in a bleach effluent from sulfite pulping were degraded by several white rot fungi, among which *T. versicolor* strain was the most efficient. Also for detoxification of environment contaminated with some phenolic compounds, *T. versicolor* was used (Bollag *et al.*, 1988). In this study, however, *C. unicolor* showed much faster growth than *T. versicolor* in PCP treated media (Fig. 2). It suggests that degradation of PCP pollutant by *C. unicolor* is more effective than that by *T. versicolor.*

At the waste sites, the accessible concentration of toxic chemicals is usually very low because of their low solubility and binding to various solid wastes surfaces. For this reason, many experiments were conducted using low concentration of PCP (Lin *et al.*, 1990). It should be noted that when the concentration of PCP in water is increased, part of this compound is either joined to solids or materialized as the insoluble sediment (Mileski *et al.*, 1988). The lethal effect of PCP was circumvented by allowing the fungus to establish a mycelial mat before PCP was added (Mileski *et al.*, 1988). The authors also reported that cultures of *P. chrysosporium* could not be inoculated with fungal spores in the presence of higher concentrations of PCP 150 μM.

3.2. Effect of Fungal Laccase on Degradation of PCP

In the case of both PCP concentrations of 50 and 100 μM, PCP dissolved in a small amount of ethanol (100 $\mu\ell$) was added to 6 day old fungal cultures in Erlenmayer flask. After 1, 3, 7 and 14 days of incubation, the HPLC analysis of PCP was performed. The results are presented in Fig. 3 and 4. After 3 days of fungal growth in the culture containing 50 μM PCP, 88.2% and 79.5% decreases of PCP were observed for *C. unicolor* and *A. biennis,* respectively (Fig. 3). The analogical data regarding 100 μM of PCP concentration are presented. In

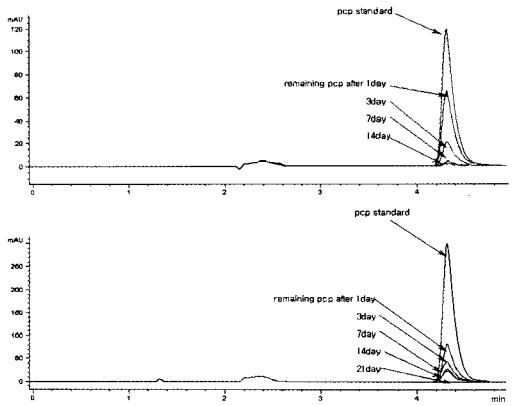


Fig. 4. HPLC chromatograms of 50 μM (upper) and 100 μM (below) PCP degradation by *A. biennis*.

this case both fungi achieved 40~55% degradation of PCP during 3 days culture. After 14 days of *C. unicolor* culture and 21 days of *A. biennis*, PCP was completely disappeared.

Bollag *et al.* (1988) reported that ethanol used to dissolve the phenolic compounds shows slight inhibitory effect. However, more noteworthy was the increase in the dry weight of the ethanol controls relative to the controls without ethanol, indicating that the ethanol was used as a carbon source by the fungus (Bollag *et al.*, 1988). The white rot fungi for the removal of chlorinated compounds from aqueous system are recommended, since these microorganisms are not dangerous for the environment, and can be cultivated on fermenter (bio-reactor) scale (Luterek *et al.*, 1998). It was reported that various lignin degrading fungi grown in bio-reactor were able to remove more than 99% PCP from synthetic waste water containing 40 to 60 mg/ℓ of this chlorophenol during one day (Wu *et al.*, 1993).

The organism often used as a model in degradation of PCP is *P. chrysosporium*, although the ability of *T. versicolor* to degrade xenobiotic chemicals has also been investigated (Bumpust and Aust, 1986). Murthy *et al.* (1979) observed that PCP could be biologically methylated to

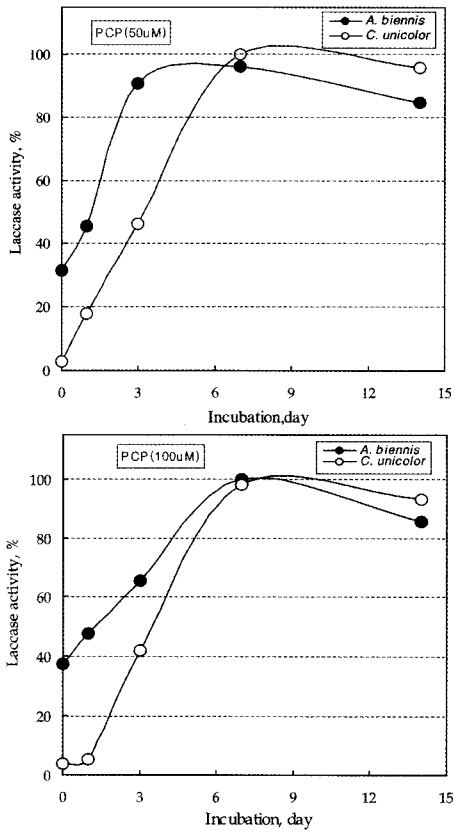


Fig. 5. The relative laccase activities of fungal culture filtrates during PCP degradation.

form pentachloroanisole (PCA). This methylated form of PCP, is the most likely produced during degradation of PCP by white-rot fungi (Badkoubi *et al.*, 1996). It was also observed that PCP could be converted back to PCP under anaerobic conditions (Badkoubi *et al.*, 1996). Other breakdown products may include 2,3,5,6-tetrachloro-2,5-cyclohexadiene-1,4-dione (TCHD) and carbon dioxide (Mileski *et al.*, 1988). The ability to mineralize organic pollutants *in vitro* is generally enhanced under culture conditions favourable for the mineralization of lignin (Bumpust and Aust, 1986). As an example, PCP would be polymerized and combined with other reactive compounds, such as coniferyl alcohol, and forms insoluble complexes in the presence of laccase

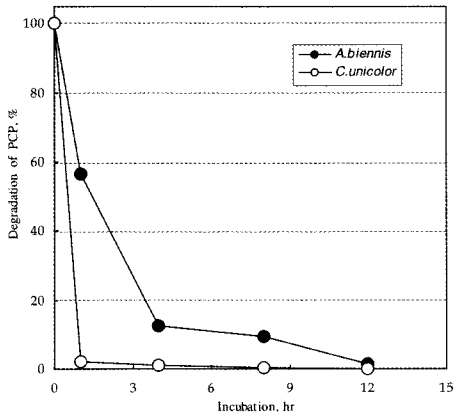


Fig. 6. The effect of 2,5-xylidine (0.2 mM) addition on PCP (50 μM) degradation.

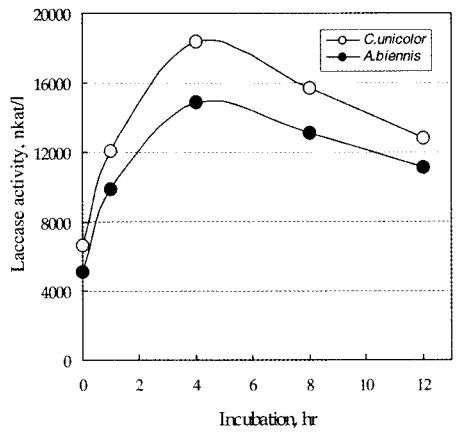


Fig. 7. The effect of 2,5-xylidine (0.2 mM) addition on laccase activity during PCP (50 μM) degradation.

(Cho *et al.*, 1999).

The relative laccase activities during fungal cultures of *A. biennis* and *C. unicolor* containing PCP are presented in Fig. 5. Fig. 5 demonstrates the changes in laccase activity during biodegradation of 50 μM and 100 μM PCP, respectively. In the case of 50 μM PCP, the activity reached to maximum at 5th and 7th days for *A. biennis* and *C. unicolor* (left), respectively. For 100 μM PCP the highest laccase activities of both fungi were at 7th day (right).

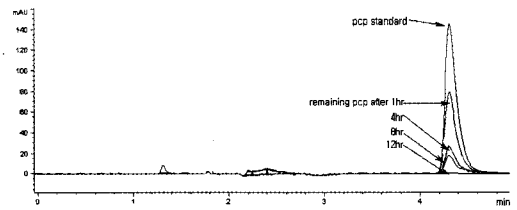


Fig. 8. HPLC chromatograms of degradation of PCP (50 μM) by *A. biennis*.

The effect of 2,5-xylidine on laccase activity and appearance of PCP in fungal cultures is shown in Fig. 6. Almost complete (98%) removal of PCP from *C. unicolor* culture was achieved within 1 hr after 2,5-xylidine addition, while at the same time the biodegradability of *A. bennis* was 58.5%. Leonowicz *et al.* already reported that among tested 17 compounds, ferulic acid, pentachlorophenol and 2,5-xylidine showed very high inducibility of laccase from tested white-rot fungi (Leonowicz *et al.*, 1997).

To demonstrate the relationship between biodegradability and laccase activity, the enzyme activity was monitored after adding 2,5-xylidine (Fig. 7). From the comparison of the results in Fig. 6 and Fig. 7, degradability (decreasing lines) and laccase activity (increasing lines) were closely related. Fig. 8 shows HPLC chromatogram of PCP degradation by the addition of 2,5-xylidine.

Fungal laccase is known from its ability to produce quinoid oligomers from some toxic chlorophenols (Minard *et al.*, 1981). The enzyme is able also to direct dechlorinate a number of chlorinated phenols. The oldest report regarded partial dechlorination of PCP by *T. versicolor* laccase (Lyr, 1963). Further Konishi and Inoue (1972) showed that PCP is primarily converted by *T. versicolor* laccase to chloranil or tetrachlorobenzoquinone and then products combine with PCP yielding benzoquinone dimers or trimers. Finally Roy-Arcand and Archibald (1991) indicated a rapid partial dechlorination produced a number of chlorinated and polychlorinated phenols by three (all known) forms of *T. versi-*

color laccase. On the other hand, several reports appeared suggesting that dehalogenation can be greatly improved by spontaneous coupling reactions with some aromatic co-substrates. In this reason the oxidative coupling process can function in two steps: firstly chlorophenols are enzymatically oxidized to free radicals or reactive quinones with aromatic co-substrates (hydrogen donors), and then subsequent oxidative coupling of the products is completed without further involvement of the enzyme (Dec and Bollag, 1994; Dec and Bollag, 1995). For example *T. versicolor* laccase incubated with guaiacol and halogenated phenols enhanced the precipitation of 4-chlorophenol, 2,4-dichlorophenol and 2,4,5-trichlorophenol by 20%, 32% and 80%, respectively, and even better result was achieved with 2,6-dimethoxyphenol which incubated with the same laccase enhanced the precipitation of 2,4,5-trichlorophenol by 98% (Roper *et al.*, 1995). It was also reported (Roy-Arcand and Archibald, 1991) that good chlorophenolic removal can be achieved also in the presence of some lignin modelling residues like macromolecular effluent from coupling processes. For example after 30 min incubation with laccase, the system removed from supernatant 86% of 2,3,4,6-tetrachlorophenol. These and other reports prompted us to further investigation of activity of fungal laccase on chlorophenols, particularly the effect of 2,5-xylidine, known as the strongest laccase inducer (Fahraeus *et al.*, 1958; Bollag and Leonowicz, 1984). The results showed that *C. unicolor* and *A. Biennis* effectively degraded PCP, and this degradation was accompanied with the increase of laccase activity. The degradation effect of PCP was greatly enhanced by 2,5-xylidine addition.

4. CONCLUSIONS

The effect of lignin degrading fungi and its laccase on the degradation of pentachlorophenol (PCP) was performed. In addition, the effect of 2,5-xylidine induced extracellular laccase on the degradation of PCP was also studied. *Abortiporus biennis, Cerrena unicolor* and *Trametes versicolor* were tested to evaluate the inhibitory effect of PCP on their growth. With the exception of *C. unicolor* and *A. biennis,* concentrated PCP higher than 100 μM completely inhibited fungal growth of *T. versicolor*. At the extremal high concentration of PCP (500 μM), only *C. unicolor* showed the highest tolerance to PCP. In the case of *A. biennis* and *C. unicolor* at 50 μM PCP concentration, about 88.2% and 79.5% of chlorophenol degradation were achieved within 3 days, respectively. By the addition of 0.2 mM 2,5-xylidine to the *C. unicolor* culture, 98% of PCP degradation was achieved within just an hour. *A. biennis* only removed 44% of PCP at the same condition. PCP was completely disappeared when laccase activities reached to the maximum. This result shows that the laccase induced by 2,5-xylidine addition influences the degradation of toxic PCP, compared with laccase alone.

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