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Removal of Chlorophenols by Fungal Laccase in the Presence of Aromatic Alcohols

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The effect of aromatic alcohols, coniferyl, sinapyl, vanillyl and iso-vanillyl alcohols, on the removal of chlorinated phenols from water environment by fungal laccases from *Cerrena unicolor* and *Rhizoctonia praticola* was studied. In optimal conditions all tested alcohols removed about 30 to 60% of chlorophenols from the supernatant, compared to that of laccase alone. *R. praticola* at pH 7.0 significantly removed more chlorophenols from supernatant than in the case of *C. unicolor* at pH 5.5. The laccase reaction is independent on the enzyme source and favours higher pH condition. Tested chlorophenols were partly coupled with aromatic alcohols and sedimented. It almost linearly depended on amounts of chlorine atoms in chlorophenols; more chlorine atoms resulted in higher yield of coupling reaction.

INTRODUCTION

Chlorophenols produced by industry on a large scale are widespread pollutants accumulating in soil and water environments (Cho *et al.*, 2001). As conventional (chemical) methods of wastewater treatment such as activated sludge or aerated lagoons are rather ineffective (Bergbauer *et al.*, 1991), recently biological method 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 in wood, show also potency to degrade many structurally diverse organic pollutants (Kang and Stevens, 1994). The removal of xenobiotic compounds from water effluent is presently understood as an enzymatic process with probably predominant function of fungal laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) (Ruggiero *et al.*, 1989; Roy-Arcand and Archibald, 1991; Dec and Bollag, 1994; Roper *et al.*, 1995; Cho *et al.*, 1999 and Cho *et al.*, 2001).

Dechlorination can be achieved with laccase (Lyr, 1963; Konishi and Inoue, 1972; Ruggiero *et al.*, 1989; Roy-Arcand and Archibald, 1991; Cho *et al.*, 2001) in the presence of some lignin residues from coupling process (Roy-Arcand and Archibald, 1991). This oxidative coupling process can function in two steps: firstly chlorophenols with aromatic co-substrates (hydrogen donors) are enzymatically oxidised to free radicals or reactive quinones, and then subsequent

oxidative coupling of the products is completed without further involvement of the enzyme (Dec and Bollag, 1994; Dec and Bollag, 1995). Chlorophenolic removal from water environment can be accomplished by coupling reaction of chlorophenols with some low molecular aromatic laccase substrates, e.g. guaiacol and 2,6-dimethoxyphenol (Dec and Bollag, 1994 and 1995; Roper *et al.*, 1995). However, since guaiacol or 2,6-dimethoxyphenol remaining in supernatants are still not indifferent, we proposed coniferyl alcohol to investigate in the coupling process more acceptable for the environment (Cho *et al.*, 1999). Coniferyl alcohol is known as a polymerizing compound during lignin biosynthesis *in vivo*, and as the substrate for *in vitro* enzymatic production of lignin modeling dehydrogenative polymer – DHP (Sterjiades *et al.*, 1996).

The objective of this study was to investigate the effect of fungal laccase with aromatic alcohols, sinapyl alcohol, vanillyl alcohol and iso-vanillyl alcohol, as the co-polymerizing factors on the removal of chlorophenols.

MATERIALS AND METHODS

Fungi

Cerrena unicolor (Bull. *ex* Fr.) Murr. No. 139 and *Rhizoctonia praticola* (De Candolle *ex* Fr.) No. 93 were from the Fungal Culture Collection (FCL) of the Department of Wood and Paper Science, Chungbuk National University, Cheongju, Korea and Department of Biochemistry, University of Lublin, Poland. The fungi were maintained on 2% (wt/vol) malt agar slants.

Culture condition

For inoculation the fungal agar plugs (ca. 0.5 cm) were cut and grown in a basal medium based on the Czapek Dox and Lindeberg media (Leonowicz *et al.*, 1984). The medium pH was adjusted to pH 5.5 and 7.0 optimal for *C. unicolor* and *R. praticola* cultures, respectively, and sterilized at 0.075 MPa for 30 min. The

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culture solution was grown static in conical flasks at 27°C till the mycelium occupied the whole surface of the liquid. The mycelial mats were collected, homogenized in a Waring Blender and used as inoculums for further incubation. The basal medium pH was adjusted to the initial pH 5.5 and 7.0, respectively, and sterilized same as described above. The shallow stationary cultures, after inoculation with 4% (vol/vol) of the mycelium homogenate, were incubated in 1000 ml flat (Roux type) flasks with 150 ml medium at 27°C. The cultures were grown up to the period corresponding to the maximum of enzyme activity in the culture fluids.

Determination of laccase activity

Laccase activity was measured at 20°C and at pH 5.5 and 7.0 on a Shimadzu UV 160 A spectrophotometer with 4-hydroxy-3,5-dimethoxy benzaldehyde azine as a substrate, but MES-NaOH buffer utilized by Leonowicz and Grzywnowicz (1981) was replaced by 0.1 M citrate-phosphate buffer (Bollag and Leonowicz, 1984). To exclude endogenous peroxide, a 10 min. preincubation (stirring) of the enzyme sample with catalase (10 mg ml⁻¹) was performed. The activity was expressed in international units, (i.e., in nkats l⁻¹, by assuming a molar absorption coefficient of 65,000 (Leonowicz and Grzywnowicz, 1981) at 525 nm for the coloured (red) reaction product.

Purification of laccase

For the purification of constitutive *T. versicolor* laccase, we used the method described earlier by Bollag and Leonowicz (1984), and for constitutive *C. unicolor* laccase the same method but modified further by Kim *et al.* (2002). The fractions of both laccases around the top of the activity (one peak each) eluted by NaCl were collected, dialysed to 0.1 M phosphate buffer, pH 5.5 and 7.0 respectively and used for further experiments.

Enzymatic treatment

For the reaction of aromatic alcohols with particular chlorophenols we directly applied the method described by Kirk and Brunow (1988), but laccase, instead of horseradish peroxidase, was applied with some other modifications as follows. All experiments were done at laboratory temperature (about 22°C). Aromatic alcohols, sinapyl alcohol, vanillyl alcohol and iso-vanillyl alcohol, were dissolved in 2 ml acetone and added (with stirring, under nitrogen) to 40 ml of degassed 0.01 M sodium phosphate buffer at pH 6.5. The clear mixture was permanently flushed with nitrogen up to the enzyme addition. The second vessel containing 10,000 nkat laccase from either *R. praticola* or *C. unicolor* in 40 ml of the same sodium phosphate buffer was 20 min stirred without flushing and 10 min preincubated (stirring) with catalase (10 mg ml⁻¹) to exclude endogenous peroxide. In the separate vessel 0.5 mM of chlorophenol was dissolved in 20 ml of the same buffer including 50,000 dpm ml⁻¹ of the respective ¹⁴C-chlorophenol. Then, to vessel containing chlorophenols, aromatic alcohols and the enzyme solutions were

either slowly added (dropping both without flushing over 20 hr with continuous stirring in darkness) or at once simultaneously added and stirred. Samples with boiled laccase were controls for determining adsorption of chlorophenols to eventually precipitated enzyme. Laccase activity was stopped by the addition of 65 μ l ml⁻¹ of 10% sodium azide (Bollag and Leonowicz, 1984). Each sediment was recovered by centrifugation (15,000 g for 30 min), washed with water (twice), evaporated in a rotary evaporator and analysed by phloroglucinol method indicating the presence of a "lignin-like" products (Trojanowski and Leonowicz, 1962). The radioactivities of the supernatants and controls were measured with Scintillation Counter as described Leonowicz *et al.* (1972).

Determination of protein

The protein content was determined according to Lowry *et al.* (1951) by using bovine serum albumin as a standard (Bollag and Leonowicz, 1984). The quantity of protein bound on sediment was calculated by subtracting the protein in the combined original liquid before laccase reaction and the supernatant and wash after incubation with the enzyme.

Chemicals

Sinapyl, vanillyl and iso-vanillyl alcohols, phloroglucinol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,5-trichlorophenol, pentachlorophenol and syringaldazine were purchased from Aldrich-Chemie (Steinheim, Germany), and then ¹⁴C-ring-labelled chlorophenols were from Pathfinder Laboratories (St Louis, Mo). Sephadex G-25 was from Pharmacia (Uppsala, Sweden) and DEAE-Cellulose 23-SS from Serva (Heidelberg, F.R.G.)

RESULTS AND DISCUSSION

According to Kirk and Brunow (1988) pH higher than 5.5 is a more convenient for polymerization of coniferyl alcohol (optimum at pH 6.5). Fig. 1 shows pH optima for *C. unicolor* and *R. praticola* laccases appearing at pH 5.5 and 7.0, respectively. Both optima seem to be more close to essential for our experiments value, than ones of laccases from other fungi existing usually between pH 3.5 and 4.5. Each laccase of those fungi usually does not show any activity at pH 7.0. As can be seen in Fig. 1, at pH 7.0 remains a meaning amount of *C. unicolor* laccase activity, at least 40% (at pH 5.5 remains around 50% of *R. praticola* laccase). These amounts are sufficient for our studies. Similar experiments with adjustment of pH were also demonstrated elsewhere (Leonowicz *et al.*, 1984)

To avoid confusing for our results adsorption of phenolic inducers to the tested enzymes, we incubated our fungi in the conditions favouring to the production of a constitutive laccase. For this reason sugar-rich medium was helpful. The enzyme is known to occur in fungi as constitutive and inducible forms (Leonowicz and Trojanowski, 1975; Leonowicz and Trojanowski,

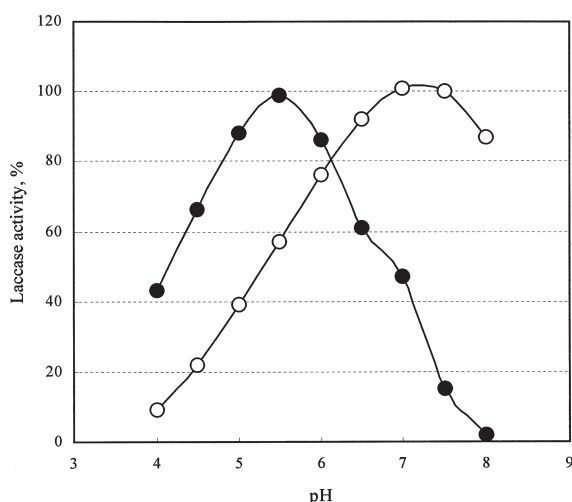


Fig. 1. The effect of pH on the activity of *C. unicolor* and *R. praticola* laccases. ● *C. unicolor*; ○ *R. praticola*

1975a; Worall *et al.*, 1986; Wahleithner *et al.*, 1996). An excess amount of glucose in liquid medium allowed for the production of constitutive laccase by the fungi, whereas the biosynthesis of the inducible enzyme forms were repressed by glucose addition (Bollag and Leonowicz, 1984; Manzanares *et al.*, 1995). In this experiment four chlorophenols were separately incubated with particular aromatic alcohol and both laccases. No significant removal of chlorophenols was observed in the controls. The results are presented in Fig. 2–Fig. 6.

Under the conditions employed, all the tested chlorophenols were partly removed from the initial solution. For example in 2 mM coniferyl alcohol (Fig. 2), the incubation with laccase of *C. unicolor* at pH 5.5 has removed 4-chlorophenol by 22%, 2,4-dichlorophenol by 48%, 2,4,5-trichlorophenol by 52% and pentachlorophenol by even 71%, compared to the level without laccase activity. The results for *R. praticola* were 34%, 57%,

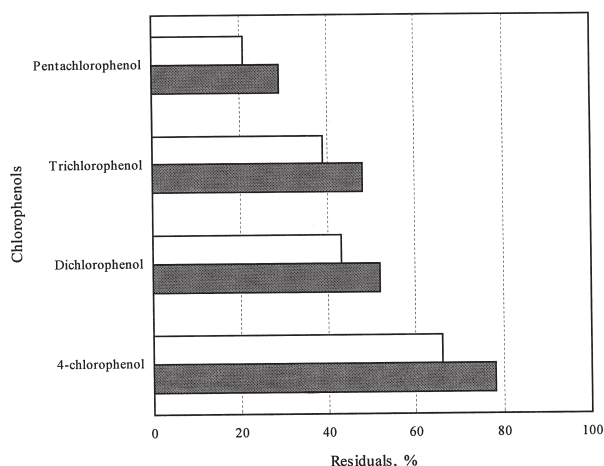


Fig. 2. Effect of coniferyl alcohol on the removal of ^{14}C -chlorophenols from the supernatants by *C. unicolor* and *R. praticola* laccases. ■ *C. unicolor*; □ *R. praticola*

61% and 79%, respectively, for particular chlorophenols. In the case of sinapyl alcohol (see Fig. 3), 4-chlorophenol by 22%, 2,4-dichlorophenol by 41%, 2,4,5-trichlorophenol by 45% and pentachlorophenol by 59% were removed for *C. unicolor*. The results for *R. praticola* were 34%, 45%, 63% and 77% removals, respectively, for particular chlorophenols (see Fig. 3). The similar results were obtained now with vanillyl alcohol (Fig. 4), and iso-vanillyl alcohol (Fig. 5). In chlorophenols, pentachlorophenol was removed very easily. In all experiments the best result was given by coniferyl alcohol, followed by sinapyl, vanillyl and iso-vanillyl alcohols (see Fig. 6).

In all experiments using either laccase form *C. unicolor* or *R. praticola*, it would be believed that tested chlorophenols were partly coupled with aromatic alcohols and sedimented during centrifugation. It almost linearly depended on amount of chlorine atoms in chlorophenols; more chlorine atoms resulted in higher

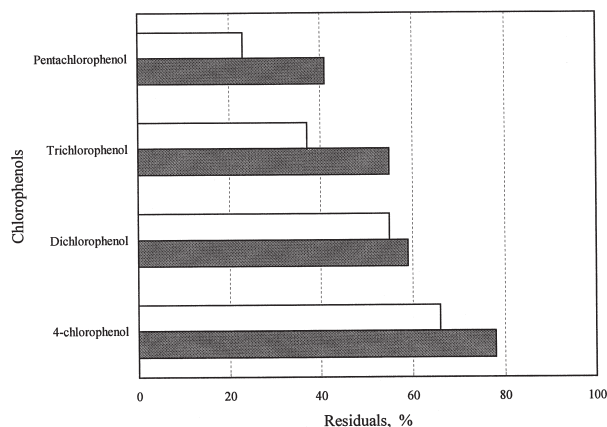


Fig. 3. Effect of sinapyl alcohol on the removal of ^{14}C -chlorophenols from the supernatants by *C. unicolor* and *R. praticola* laccases. ■ *C. unicolor*; □ *R. praticola*

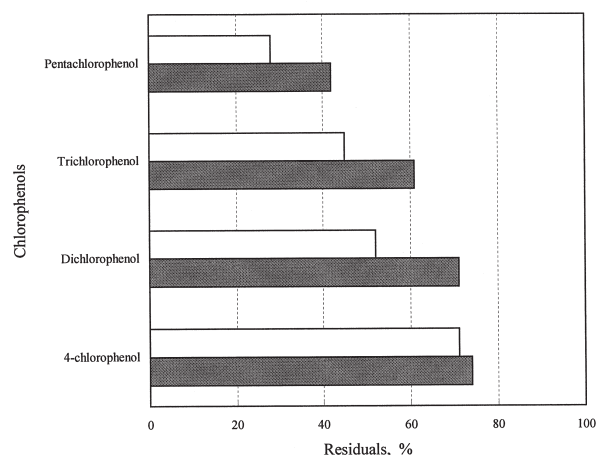


Fig. 4. Effect of vanillyl alcohol on the removal of ^{14}C -chlorophenols from the supernatants by *C. unicolor* and *R. praticola* laccases. ■ *C. unicolor*; □ *R. praticola*

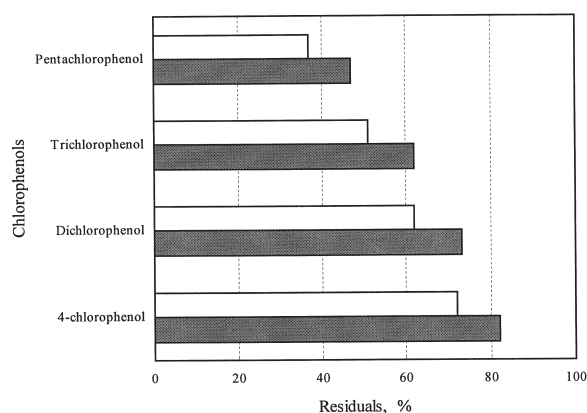


Fig. 5. Effect of iso-vanillyl alcohol on the removal of ^{14}C -chlorophenols from the supernatants by *C. unicolor* and *R. praticola* laccases. ■ *C. unicolor*; □ *R. praticola*

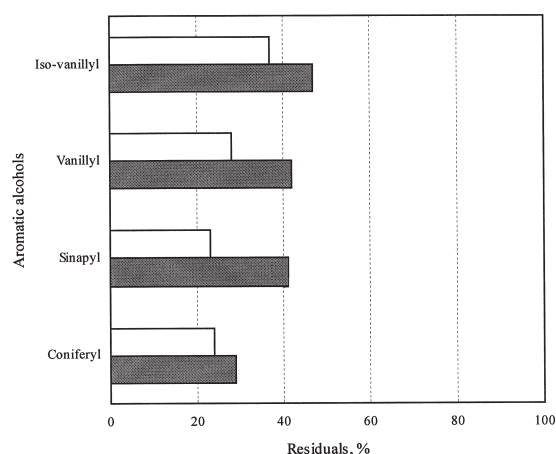


Fig. 6. Effect of *C. unicolor* and *R. praticola* laccases and aromatic alcohols on the removal of ^{14}C -chlorophenol from the supernatants. ■ *C. unicolor*; □ *R. praticola*

yield of coupling reaction products. This relationship repeated in the experiments with both fungi (Fig. 2). However, *R. praticola* at pH 7.0 significantly removed more chlorophenols from supernatant than in the case of *C. unicolor* at pH 5.5. These results were a little lower removal in comparison with results obtained earlier for coniferyl alcohol (Cho *et al.*, 1999), which showed about 40% of 4-chlorophenol, 54% of 2,4-dichlorophenol, 60% of 2,4,5-trichlorophenol and even 76% of pentachlorophenol, compared to the level without laccase activity.

Any meaningful differences in the results were not noticed, what confirms earlier results, where activity of two different laccases on vanillic and syringic acids was investigated (Leonowicz *et al.*, 1984). In that case it was unequivocally stated that the laccase reaction is independent on the enzyme source and favours higher pH conditions (Leonowicz *et al.*, 1984). For confirmation of this fact, we followed the experiments with applying pH 7.0 for laccase of *C. unicolor* and 5.5 for

the enzyme isolated from *R. praticola*. The results were almost similar as earlier. When we tested laccases of *C. unicolor* at pH 5.5 and *R. praticola* at pH 7.0, it confirms independency of reaction from source of the enzyme and dependency from pH.

The laccase enzyme is known from its ability to production of quinoid oligomers from some toxic chlorophenols (Minard *et al.*, 1981). The enzyme is also able to directly dechlorinate a number of chlorinated phenols. The oldest report regarded partial dechlorination of pentachlorophenol by *Trametes versicolor* laccase (Lyr, 1963). Furthermore Konishi and Inoue (1972) reported that pentachlorophenol is primarily converted by *Coriolus versicolor* laccase to chloranil or tetrachloro-benzochinone, and then products combine with PCP yielding benzoquinone dimers or trimers. Finally Roy-Arcand and Archibald (1991) indicated a rapid partial dechlorination of many chlorinated and polychlorinated phenols by three laccases from *Trametes versicolor*. Presented our earlier report (Cho *et al.*, 1999) and this report confirming the coupling reaction point to one more method for removal of toxic chlorophenols from water environment. In both experiments either *C. unicolor* or *R. praticola* laccase could be applied. Our present experiments confirmed also the thesis that coupling reactions prefer higher pH. These aromatic alcohol compounds, especially iso-vanillyl alcohol, are much cheaper than coniferyl alcohol. Additively, using vanillyl or iso-vanillyl alcohol allows for saving money. In our coupling experiments we obtained the sediment, which was dissolved in the mixture of ethyl alcohol and ethylene chloride, and gave a red colour with phloroglucinol-HCl. This positive result of phloroglucinol test shows the coupling reaction of some aldehyde group of the sediment with phloroglucinol (Trojanowski and Leonowicz, 1962). Such polymeric, insoluble sediments produced during coupling reaction may be used for enriching of soil humus.

CONCLUSIONS

This study was carried out to investigate the effect of fungal laccase with aromatic alcohols, sinapyl alcohol, vanillyl alcohol and iso-vanillyl alcohol, as the co-polymerizing factors, on the removal of chlorophenols. In optimal conditions all tested alcohols removed about 30 to 60% of chlorophenols from the supernatant, compared to that of laccase alone. *R. praticola* at pH 7.0 significantly removed more chlorophenols from supernatant than in the case of *C. unicolor* at pH 5.5. The laccase reaction is independent on the enzyme source and favours higher pH condition. Tested chlorophenols were partly coupled with aromatic alcohols and sedimented. It almost linearly depended on amounts of chlorine atoms in chlorophenols; more chlorine atoms resulted in higher yield of coupling reaction.

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