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Effect of coniferyl alcohol addition on removal of chlorophenols from water effluent by fungal laccase

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Abstract The effect of coniferyl alcohol on removal of chlorinated phenols from a water environment by *Rhizoctonia praticola* and *Cerrena unicolor* laccases was studied. At optimal conditions in which 7mM coniferyl alcohol and laccase were added to chlorinated phenols over 20h, about 34% of the radioactivity of 4-chlorophenol, 57% of 2,4-dichlorophenol, 66% of 2,4,5-trichlorophenol, and 85% of pentachlorophenol were removed from the supernatants, compared to the level without laccase activity. After 12-h incubation periods at the optimal concentrations of coniferyl alcohol and laccase (added simultaneously), the fast first phase of chlorophenol removal was complete in 1h, and eventually coniferyl alcohol enhanced the removal of 4-chlorophenol by 40%, 2,4-dichlorophenol by 54%, 2,4,5-trichlorophenol by 60%, and pentachlorophenol by 76%.

Key words Chlorophenols · Coniferyl alcohol · Laccase · *Rhizoctonia praticola* · *Cerrena unicolor*

Introduction

Fungal laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2), extracellularly produced by several wood rotting fungi,[1] seems to be applicable to various biotechnological processes.[2–4] Among possible applications, the enzyme is considered to be a dechlorinating factor.[5] Its broad activity on hydrogen donors provides opportunities for removing certain phenolic and other aromatic compounds from natural and industrial wastewaters.[6–8]

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The oxidation of phenolic compounds generates phenoxy radicals and quinoid intermediates that are subsequently transformed to dimers and insoluble polymers with aromatic amines such as chloroamines.[9] After sedimentation, such polymers may be removed from the water environment.[10] Substrates that react readily with the enzyme enhance the removal of such aromatic, amines, which are less reactive.[11]

Roper et al.[12] proposed the decontamination of industrial wastewaters from chlorophenols by copolymerization with typical laccase hydrogen donors such as guaiacol or 2,6-dimethoxyphenol. But, as these substances are still toxic for water, the objective of this study was to investigate the effect of addition of coniferyl alcohol on the removal of chlorophenols in the effluents. Coniferyl alcohol is more acceptable in the environment than phenolic compounds of outside origin. The applied concentration of coniferyl alcohol was five times lower than the others.[5,12] This compound also is known as a constituent of lignified tissue[12] during lignin biosynthesis in vivo and as the substrate for in vitro enzymatic production of lignin model dehydrogenative polymer (DHP).[13]

Materials and methods

Biological material

Rhizoctonia praticola (De Candelle ex Fr.) and *Cerrena unicolor* (Bull. ex Fr.) Murr. were obtained from the Fungal Culture Collections of the Department of Biochemistry, University of Lublin, Poland (FCL) and the Department of Forest Products, Chungbuk National University, Cheongju, Korea (FCC). The fungi were maintained on 2% (w/v) malt agar slants.

Culture conditions

For inoculation the fungal agar plugs (ca. 0.5cm) were cut and grown in a basal medium based on the Czapek Dox and

Lindeberg media.[14] The medium pH was adjusted by phosphate to the optimal pH 6.5 and 5.5 for *R. praticola* and *C. unicolor* cultures, respectively. They were sterilized at 0.075 MPa for 30 min. The cultures were grown in static conical flasks at 27°C till the mycelia occupied the whole surface of the liquid. The mycelial mats were collected, homogenized in a Waring Blender, and used as inoculum for further incubation. The shallow stationary cultures, after inoculation with 4% (v/v) of the mycelia homogenates, were incubated in 1000-ml flat (Roux type) flasks with 150 ml medium at 27°C.

Determination of laccase activity

Laccase activity was measured at 20°C and pH 6.5 on a Shimadzu UV 160A spectrophotometer with syringaldazine as a substrate; the MES [2(*N*-morpholino) ethane sulphonic acid]-NaOH buffer utilized by Leonowicz and Grzywnowicz[15] was replaced by 0.1 M citrate – phosphate buffer.[1] To exclude endogenous peroxide, a 10-min preincubation (stirring) of the enzyme sample with catalase (10 mg/ml) was performed. The activity was expressed in international units (i.e., nkat/l) by assuming a molar absorption coefficient of 65000[15] at 525 nm for the colored (red) reaction product. It was calculated as follows:

$$\text{Activity}\left(\text{nkat/l}\right) = \frac{\Delta A_{525} \times \text{total volume}\left(\text{ml}\right) \times 10^9}{\varepsilon \times dt \times \text{sample volume}\left(\text{ml}\right)}$$

where kat is mol/s; ε (syringaldazine) is 65000; dt is seconds (= 60); total volume is 1 ml; and sample volume is 0.1 ml.

Purification of laccase

For the purification of laccase the method described by Bollag and Leonowicz[1] was applied with modifications as follows. The fungal cultures with the highest activity level were filtered through Miracloth (Calbiochem). The filtrate was desalted on a Sephadex G-25 column and the enzyme concentrated on the Amicon ultrafiltration system to about one-tenth of the volume using a filter type PTGC with a pore size 10000 NMWL at 4°C. The concentrated preparations were applied to a DEAE-cellulose 23-SS column (1.6 × 20 cm) equilibrated with 5 mM Tris-HCl buffer pH 6.5. The unwanted proteins were washed out with the initial buffer, and the enzymes were eluted with a linear gradient 0–0.5 M NaCl in 5 mM Tris-HCl buffer pH 6.5. Fractions of 4 ml were collected at a flow rate of about 50 ml/h. The fractions around the top of the activity (one peak) eluted by NaCl were collected, dialyzed to 0.1 M phosphate buffer pH 6.5, and used for experiments.

Enzymatic copolymerization

Laccase was applied for the copolymerization of coniferyl alcohol with particular chlorophenols,[16] and some other modifications were made as follows. All work on polymerization was done at laboratory temperature (about 22°C). Coniferyl alcohol was dissolved in 2 ml acetone and added (with stirring, under nitrogen) to 40 ml of degassed 0.01 M sodium phosphate buffer pH 6.5. The clear mixture was flushed with nitrogen until addition of the enzyme. The second vessel containing 10000 nkat of either *R. praticola* or *C. unicolor* laccase in 40 ml of the same sodium phosphate buffer was stirred 20 min without flushing and preincubated (stirring) 10 min with catalase (10 mg/ml) to exclude endogenous peroxide. In the separate vessel 0.5 mM of chlorinated phenol was dissolved in 20 ml of the same buffer including 50000 dpm/ml of the respective ^{14}C-chlorophenol. Then, to the vessel containing chlorophenol, coniferyl alcohol and the enzyme solutions were either slowly added (dropping both without flushing over 20 h with continuous stirring in darkness) or at once simultaneously and stirred. Samples with boiled laccase were controls for determining the adsorption of chlorophenols to eventually precipitated enzyme. Laccase activity was stopped by adding 65 μl/ml (to 0.01 mM) of 10% sodium azide according to Bollag and Leonowicz.[1] The sediment was recovered by centrifugation (15000 *g* for 30 min), washed with water (twice), evaporated in a rotary evaporator, and analyzed by phloroglucinol method indicating the presence of "lignin-like" products as described by Trojanowski and Leonowicz.[17] The radioactivity of the supernatants and controls, and sediment [after dissolving it in the mixture of ethyl alcohol and ethylene chloride (1:1 v/v)] was counted in the toluene scintillation fluid as described by Leonowicz et al.[18] Samples deprived of chlorophenols served as a control of laccase polymerizing activity on coniferyl alcohol. All experiments with radioactive chlorophenols were done threefold, and the final results were presented as the average of these determination.

Determination of protein

The protein content was determined according to Lowry et al.[19] using bovine serum albumin as the standard.[1] The quantity of protein bound on sediment was calculated by subtracting the proteins in the combined original liquid before the laccase reaction and the supernatant and washed after incubation with the enzyme.

Chemicals

Coniferyl alcohol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,5-trichlorophenol, pentachlorophenol, 2,6-dimethoxyphenol, and syringaldazine were purchased from Aldrich-Chemie (Steinheim, Germany); the ^{14}C-ring-labeled chlorophenols were from Pathfinder Laboratories (St Louis, MO, USA); Sephadex G-25 was from Pharmacia (Uppsala, Sweden); and DEAE-cellulose 23-SS was from Serva (Heidelberg, Germany).

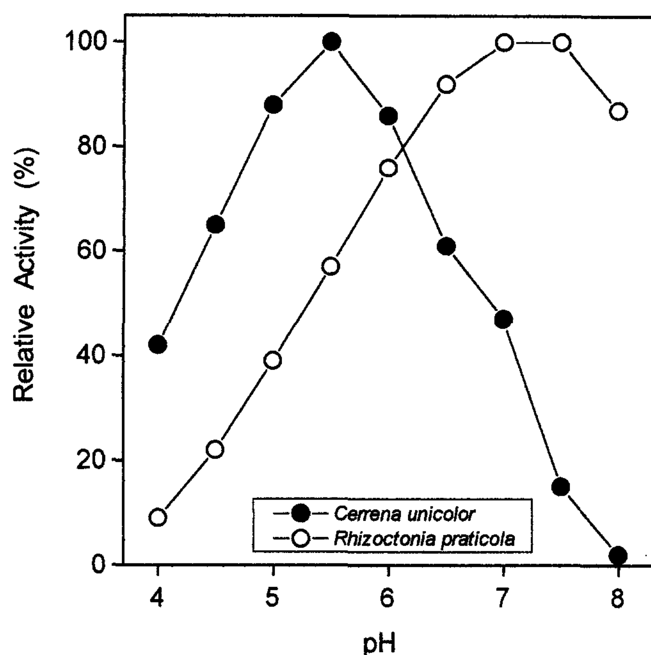

Fig. 1. Effect of pH on the activity of *Cerrena unicolor* and *Rhizoctonia praticolor* laccases

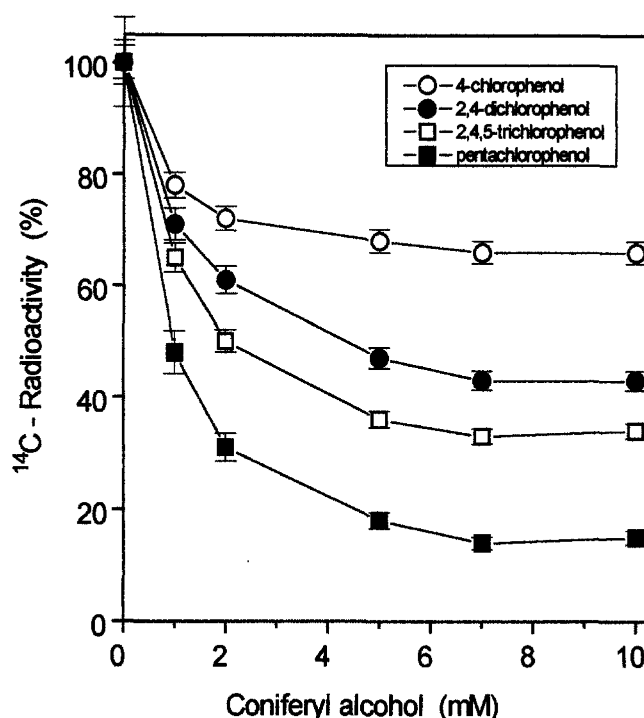

Fig. 2. Effect of coniferyl alcohol concentration on the removal of ^{14}C chlorophenols from the supernatants by *Rhizoctonia praticolor* laccase after a 20 h incubation

Results and discussion

Fungal laccase that has high activity at pH optima possibly close to 6.5 was chosen because this pH is appropriate to polymerization of coniferyl alcohol.[16] Figure 1 shows the pH optima for *R. praticola* and *C. unicolor* laccases: pH 6.8 and 5.5, respectively. Both optima seem to be closer to our experimental value than the pH of laccases of other fungi, which are usually between pH 3.5 and 4.5.[1,20] The laccase of those fungi usually does not show any activity at pH 6.5. As can be seen in the Fig. 1, a meaningful amount of laccase activity at pH 6.5 remains: at least 90% and 50% for *R. praticola* and *C. unicolor*, respectively. These amounts are sufficient for our studies. Similar experiments with pH adjustment were also demonstrated elsewhere.[14]

To avoid confusion about adsorption of phenolic inducers to the enzyme, both fungi were incubated at conditions that favored production of a constitutive laccase. For this purpose a sugar-rich medium was helpful. The enzyme is known to occur in fungi in constitutive and inducible forms according to the contents of carbon sources.[20–23] An excess of saccharose or glucose in liquid medium allowed constitutive production of laccase by our fungi, whereas biosynthesis of the induced enzyme forms was repressed by sugar.[1,4]

In the first experiment, four radioactive chlorophenols were separately incubated with various amounts of coniferyl alcohol and laccase from *R. praticola* (20 h, with stirring). The results are presented in Fig. 2. Under the conditions employed, all the tested chlorophenols were removed from the initial solutions. At an optimal (7 mM) concentration of coniferyl alcohol and laccase, we simultaneously added chlorinated phenols with continuous stirring for 24 h to the incubation system; this process removed from the supernatants about 34% of the radioactivity of 4-chlorophenol, 57% of that of 2,4-dichlorophenol, 66% of that of 2,4,5-trichlorophenol, and 85% of that of pentachlorophenol (PCP), compared to the level without laccase activity. The optimum reaction occurred with 7 mM coniferyl alcohol, but a significant result was seen as well with much lower concentrations of this substrate (even 2 mM).

In the second experiment various time periods for the reaction of coniferyl alcohol with laccase from *C. unicolor* and chlorophenols were employed. All constituents of the polymerizing process were immediately mixed and incubated for 12 h with stirring. For all the incubations we used the optimal 7 mM coniferyl alcohol based on the result from Fig. 2. The results are presented in Fig. 3. In this case coniferyl alcohol enhanced the removal of 4-chlorophenol by 40%, 2,4-dichlorophenol by 54%, 2,4,5-trichlorophenol by 60%, and PCP by 76%. The time course for the reaction of coniferyl alcohol and particularly chlorophenol with laccase showed that after a fast initial decrease the change in concentration of radioactivity in the supernatant slowed.

The fast first phase finished after 1 h of incubation. Further incubation produced only insignificant changes, and the level of radioactivity practically reached a plateau. The exception was 4-chlorophenol, where a little more removal of radioactivity between 1 and 12 h of incubation was noted. Comparing the results presented in Figs. 2 and 3, it can be easily seen that the optimal rate of radioactivity removal in

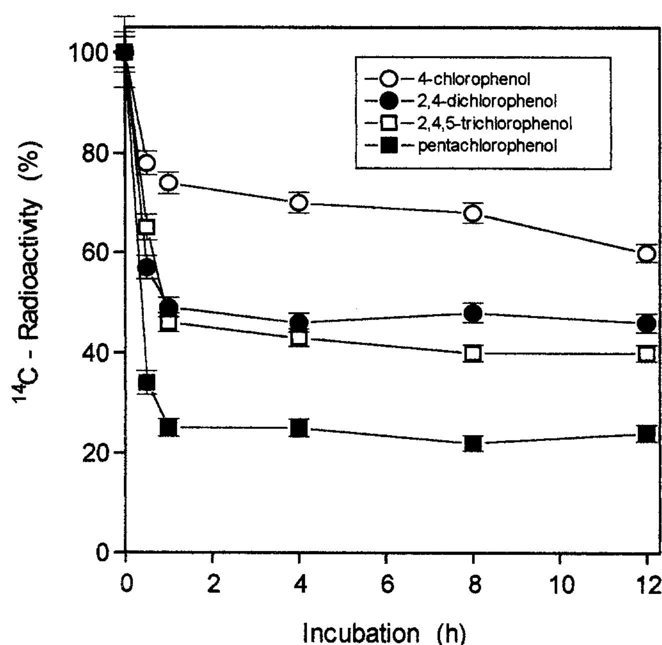

Fig. 3. Time course for the removal of ^{14}C chlorophenols from supernatants by laccase from *Cerrena unicolor* after incubation with coniferyl alcohol

the second experiment was lower than that in the first experiment, which probably was influenced by the method used to mix the constituents. Therefore the slow combination of constituents is more effective than the rapid one. The difference, however, is not significant.

In both experiments either *R. praticola* or *C. unicolor* laccases could be applied. The total radioactivity calculated by summing the radioactivities of the supernatant and the sediment were in each case nearly 100% of the initial ones. No meaningful differences in the results were noted, which confirms earlier studies, where activities of two different laccases on vanillic and syringic acids were investigated.[14] In this case it was unequivocally stated that the course of the laccase reaction is independent on the enzyme source and favors pH conditions.

Fungal laccase is known for its ability to produce quinoid oligomers from some toxic chlorophenols.[24] The enzyme is able also to direct dechlorination of a number of chlorinated phenols. The oldest report was on the partial dechlorination of PCP by *Trametes versicolor* laccase.[25] Konishi and Inoue[26] later showed that PCP is primarily converted by *T. versicolor* laccase to chloranil or tetrachlorobenzoquinone, with the products combining with PCP to yield benzoquinone dimers or trimers. Finally, Roy-Arcand and Archibald[5] indicated rapid partial dechlorination of a number of chlorinated and polychlorinated phenols by three (all known) isoforms of *T. versicolor* laccase. Other reports suggested, in contrast, that dehalogenation can be greatly improved by spontaneous coupling reactions with some aromatic co-substrates. The oxidative coupling process can function in two steps: First, chlorophenols with nonchlorinated phenolic co-substrates (hydrogen donors) are enzymatically oxidized to free radicals or reactive quinones; then subsequent oxidative coupling of the products is completed without further involvement of the enzyme.[27,28] 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 results were achieved with 2,6-dimethoxyphenol, which when incubated with the same laccase enhanced precipitation of 2,4,5-trichlorophenol by 98%.[12] It was also reported by Roy-Arcand and Archibald[5] that good chlorophenol removal from the effluent can be achieved by a coupling process in the presence of some lignin model compounds. For example, after a 30-min incubation with laccase the system removed 86% of 2,3,4,6-tetrachlorophenol from the supernatant. These reports prompted us to use coniferyl alcohol as a co-substrate. The free radicals working in the system are coupled by nonenzymatic condensation, leading to "lignin-like" dehydrogenative polymer (DHP) formation, which was confirmed by the phloroglucinol-HCl reaction.[13]

In our coupling experiments we obtained sediment, which after dissolving in the mixture of ethyl alcohol and ethylene chloride gave a red color with phloroglucinol-HCl.[17] These results, which were similar to the coupling experiments of Roper et al.,[12] point to one more method for removing toxic chlorophenols from the water environment. The sediment produced during the coupling reaction may enrich soil humus into aromatic substrates and can be directly removed by fungal laccase,[5] which is present in soil.[29]

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