Removal of chlorophenols from wastewater by immobilized horseradish peroxidase

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1.Introduction

A number of chlorinated compounds are present in wastewater from pulp and paper mills. Most of the compounds are toxic, and can not easily be removed by conventional wastewater treatment methods such as coagulation and sedimentation or biological treatment. The applicable methods for the removal of the compounds, adsorption to activated carbon, chemical oxidation and membranes, suffer from things defect such as high cost, concentration and formation of other hazardous products. As an alternative method, wastewater treatments using enzymes such as tyrosinase[1-4], peroxidase[5-10], and laccase[11-13] have been attracting a great deal of interest lately. Horseradish peroxidase was shown to be able to remove a variety of phenols and aromatic amines from an aqueous solution[5,6] and to decolorize from phenolic industrial effluents[10]. Recently, lignin peroxidase and manganese peroxidase of basidiomycetes, which play an important role in the breakdown lignin, are reported to be effective for treatment of pulp-bleaching plant effluents[14-16]. Enzymatic treatment is based on the principle that enzymes catalyze the oxidation of phenols to form corresponding radicals or quinones, and they are removed by a precipitate resulting from their polymerization.

Enzyme immobilization offers advantages such as high storage stability and better control of the catalytic process. In addition, immobilization allows for operational stability and no contamination of the solution treated by enzymes, because the immobilized enzymes can be easily separated from the solution. Horseradish peroxidase was immobilized on controlled porous glass (CPG) and 300-400 units/g of immobilized peroxidase was obtained[19]. Siddique et al.[7] reported on the removal of *p*-chlorophenol from an aqueous solution by horseradish peroxidase immobilized on three different reactor matrices. Several immobilization methods (physical adsorption, covalent bonding, crosslinking, inclusion, and encapsulation) have been developed to immobilize enzymes. Physical adsorption is the most cost-effective and simplest of all immobilization methods. A variety of supports can be used to immobilize enzymes. The use of magnetic particles, as a support, has the advantage of being easily separable from the solution through the use of magnetic devices. In the present work, removal of chlorophenols in an aqueous solution by immobilized peroxidase was investigated.

2. Materials and Methods

Chemicals

All chemicals are commercially available and of reagent grade. Phenol was obtained from Wako Chemicals (Tokyo, Japan); *p*-chlorophenol, 2,4-dichlorophenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Pentachlorophenol was recrystallized twice from benzene.

Enzyme

Horseradish peroxidase (EC 1.11.1.7) was purchased from Wako Chemicals (Tokyo, Japan) and had a specific activity of 100 units/mg. Peroxidase activity was determined from a change in the optical density ($A_{405\ nm}$) in a reaction mixture containing 2,2'-azino-bis[3-ethyl-benzothiazoline-(6)-sulphonic acid] diammonium salt from Wako Chemicals [18]. The change was determined by using a spectrophotometer (Jasco Ubest-55, Japan Spectroscopic, Tokyo, Japan). One unit of peroxidase activity was defined from an increase in A_{405} nm per minute at pH 6.0 at 25 °C in a 3 mL reaction mixture.

Immobilization of peroxidase

The immobilization of peroxidase on magnetite was carried out in an aqueous solution containing peroxidase. The enzyme was dissolved in pure water, and stirred with magnetite (500 mg) at 25 °C for 15 h. The immobilized protein was calculated from the difference between peroxidase concentration before and after immobilization. Protein concentration was determined by the Berden method [19].

Incubation conditions

Reactions of each phenol with peroxidase in the presence of hydrogen peroxide were performed at 25°C in 7 mL of a 10 mM phosphate buffer (pH 7.0) as follows: a phenol solution was first added to a phosphate buffer solution containing immobilized peroxidase, followed by an addition of hydrogen peroxide. Phenol solution was incubated under aerobic conditions using a stirrer (Tokyo Rika Kikai MDC-RT, Tokyo, Japan). A model wastewater was prepared containing various chlorophenols: *p*-chlorophenol (200 μM), 2,4-dichlorophenol (200 μM), 2,4,5-trichlorophenol (100 μM), 2,4,6-trichlorophenol (100 μ M), 2,3,4,6-tetrachlorophenol (20 μM) and pentachlorophenol (10 μM), and treated by immobilized peroxidase in the presence of hydrogen peroxide. The model wastewater was incubated by the same conditions as that of phenol, except for the use of the phosphate buffer, pH 5.5. After a prescribed time, the sample was withdrawn and assayed for phenols, total organic carbon (TOC), and adsorbable organic halogen (AOX).

Analysis

The determination of TOC was made on a Shimadzu TOC analyzer Model 500 (Tokyo, Japan), and AOX was determined by TOX-10Σ (Mitsubishi Kasei Co., Tokyo, Japan). The disappearance of each phenol was monitored by high performance liquid chromatography using a Jasco PU-980 (Japan Spectroscopic) provided with an UV detector (Jasco UV-970) and an integrator (Jasco 807-IT). A reverse phase column, Cosmosil C18-AR (5 μ, 4.6 mm i.d. x 25 cm, Nakarai tesque, Kyoto, Japan), was used, and the mobile phase, flow rate 1.0 mL/min, consisted of methanol and water containing 0.08 % H_3PO_4 50:50 v/v. In the analysis of model wastewater containing chlorophenols, the initial mobile phase consisted of

50:50 v/v methanol and 0.08 % H_3PO_4 which was brought to 90:10 v/v by 20 min and held for another 20 min. After a specified time, 20 μl of reaction solution was injected. The absorbance at 400 nm of reaction solution was determined by using the spectrophotometer.

3. Result and discussion

Figure 1 shows the difference between the removal of phenol with immobilized peroxidase and soluble peroxidase. By the immobilized peroxidase inactivated in boiling water for 10 min, no reduction in phenol took place. The results clearly showed that the phenol reduction by immobilized peroxidase was caused only by an enzymatic reaction, not by adsorption on magnetite or enzyme. The concentration of phenol decreased with time, and the immobilized enzyme was much more effective than the soluble enzyme. In the case of soluble peroxidase, almost no decrease was observed after 3 min. This was due to the inactivation of peroxidase caused by the reaction with reaction products such as quinones or radicals.

Removal of each chlorophenol from the wastewater by peroxidase can be seen in Table 1. We used the same enzyme activity (0.2 units/mL) for both these cases; soluble and immobilized enzyme. The results indicate, as well as the results described previously, that immobilized enzyme was more effective than the soluble one, and each phenol was removed to almost 100%. For soluble peroxidase, 2,4,6-trichlorophenol was more reactive than 2,4,5-trichlorophenol. Such an effect on the chlorinated position of phenol could not observe in immobilized peroxidase.

Table 1 Removal of each chlorophenol from model wastewater by soluble and immobilized horseradish peroxidase.

	Concentration (μM)	Removal(%) Free H.P.	Removal(%) Immobilized H.P.
p-Chlorophenol	200	58	100
2,4-Dichlorophenol	200	82	100
2,4,5-Trichlorophenol	100	36	99
2,4,6-Trichlorophenol	100	97	98
2,3,4,6-Tetrachlorophenol	20	81	99
Pentachlorophenol	10	55	97

H.P.: Horseradish peroxidase, Enzyme activity: 0.2 units/mL, H_2O_2: 1 mM, pH: 5.5, Incubation time: 30 min.

TOC and AOX of the model wastewater were 43 and 45 ppm, respectively. In the soluble peroxidase of 0.2 units/mL, TOC and AOX could not be removed, because no precipitation occurred. Phenol removal by enzyme-catalyzed polymerization was investigated. However, there are almost no reports on contamination due to the remaining soluble enzyme and non-precipitated products in the aquatic solution after the peroxidase treatment. The evaluation of TOC and AOX removal provides very important information on using enzymes for waste-

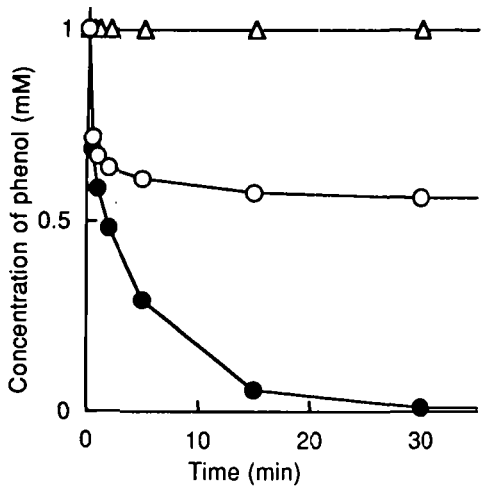

Fig.1 Removal of phenol by soluble and immobilized horseradish peroxidase

Inactivated enzyme
Soluble enzyme
Immobilized enzyme

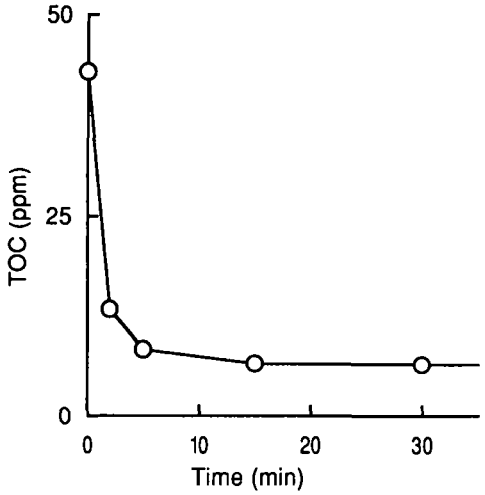

Fig.2 Removal of TOC from model waste water by immobilized horseradise peroxidase.

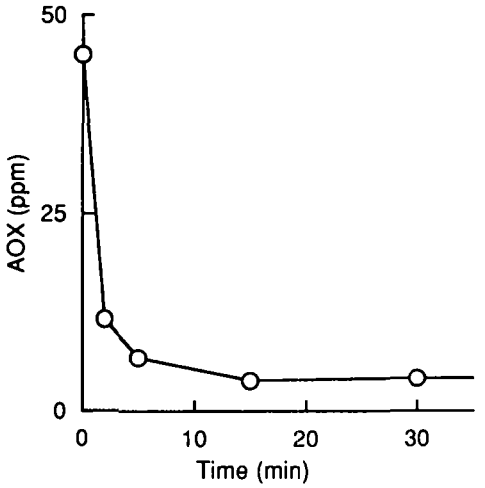

Fig. 3 Removal of AOX from model waste water by immobilized horseradish peroxidase.

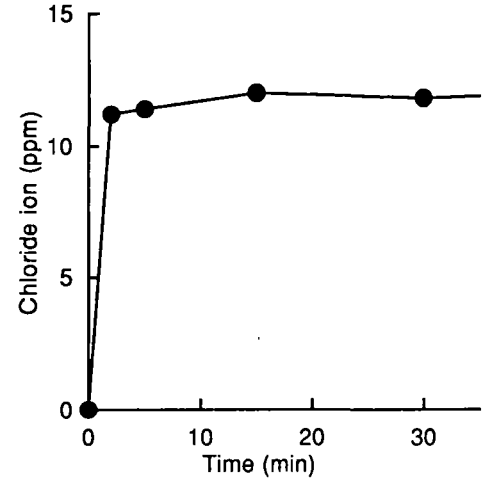

Fig. 4 Chloride ion released from chlorophenols during incubation with horseradish peroxidase.

water treatment.

Removal of TOC and AOX from model wastewater containing various chlorophenols by immobilized peroxidase is shown in Figures 2 and 3. As described previously, no release of enzymes occurred from the immobilized peroxidase. In 10 min, about 90% of TOC and AOX was found to be removed. This indicates that the reaction products formed from each chlorophenol were linked to enzymes immobilized on magnetite, because they were not adsorbed on magnetite. This study indicates that when you use immobilized peroxidase, chlorophenols can be removed without any coagulant.

Chloride ions are known to be released during the enzymatic coupling processes of chlorophenols[18]. Figure 4 shows the concentration of chloride ions released from chlorophenols during enzymatic treatment. The concentration of released chloride ions was 12 ppm, and it was found to constitute about 25% of AOX. An increase in chloride ions took place at almost the same time as the decrease in TOC and AOX. Dec and Bollag[19] showed that the processes of dechlorination coincided almost exactly with the course of the 2,4-dichlorophenol disappearance. They also showed that the maximum amount of chloride ions released was 20% of the total chlorine initially associated with 2,4-dichlorophenol.

In conclusion, the immobilized peroxidase can very effectively remove phenols because of the binding of colored reaction products to the immobilized enzyme. In the treatment of model wastewater containing chlorophenols, more than 90% of TOC and AOX were found to be removed by immobilized peroxidase.

4. References

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