A NEW METHOD TO REMOVE PENTACHLOROPHENOL CATALYTICALLY IN WASTEWATER BY IMMOBILIZED HORSERADISH PEROXIDASE

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ABSTRACT

A new technique that horseradish peroxidase (HRP) immobilized on the carrier of Fe_3O_4 adsorption and gelatin embedding was applied to catalyze the removal of pentachlorophenol (PCP), one of organic chlorine pesticides. Experiments were carried out to determine the optimal reaction conditions: The dosage ratio of HRP: Fe_3O_4: gelatin was 95 U: 1.0 g: 10 mL (10–20%); the concentration of glutaraldehyde was 0.5%; the cross-linking time was 30 minutes. Under these conditions, the enzymatic activity of immobilized HRP was 1.1 $U \cdot g^{-1}$ (wet weight). The removal efficiencies of PCP by immobilized HRP and free HRP were compared. The reaction equilibrium was achieved in average 30 min, but immobilized HRP saw a broader pH range (4–6) with higher maximum removal efficiency (41% at pH 5) and a lower K_m compared with free HRP. The amount of PCP removed by immobilized HRP increased with the initial concentration of PCP rising, although the removal efficiency decreased from 39.7% to 24.4%. The percentage of removed PCP remained>39% when HRP (0.05 $U \cdot mL^{-1}$) was used repeatedly for 7 rounds. This work provided a method for further studies to deal with endocrine-disrupting chemicals in wastewater.

Keywords: Cross-linking, Horseradish peroxidase, Immobilization, Pentachlorophenol, Catalytic removal.

1. Introduction

Pentachlorophenol (PCP), is a kind of phenol compounds with high toxicity. It is widely used for the preservation of schistosomiasis and wood rot (USEPA, 1997). PCP is an endocrine-disrupting chemical and persistent organic pollutant (Jekat *et al*.， 1994; Dimich *et al*., 1996; Gerhard *et al*., 1999). It not only interferes with the endocrine systems of fish and mamals (Zhang *et al*., 2004; Rawlings *et al*., 1998; Beard *et al*., 1999), but also has obvious estrogenic effects on humans (Danzo, 1997; Tran *et al*., 1996). Thanks to decades of production and overuse, pollution of PCP is serious in soil, sediment, vegetables, especially exceeds the acceptable hazard level in wastewaters in China (Chang and Jin, 2002; Zhang *et al*., 2001). Therefore, PCP is a great threat to the environment and human health.

Catalysis of enzyme is regarded as one of the effective degradation pathways to remove PCP (Moeder *et al*., 2004; Xun and Webster， 2004; Hublik and Schinner, 2000). Horseradish peroxidase (HRP) was applied to degrade PCP (Choi *et al*., 1999; Zhang and Nicell, 2000). But water soluble enzyme belongs to one-time consumption with low recoverability, the technique immobilization was born (Song *et al*., 2003; Kim and Moon, 2005). Entezari and Petrier (2003) combined ultrasonic wave and HRP to see the degradation effeciency, showing the combination method was superior to the single one.

In this study, Fe_3O_4 adsorption and gelatin embedding technique is first applied to prepare immobilized HRP to catalyze the degradation of PCP. The factors that influence this process, i.e., time, pH, enzyme dosage, and initial concentration of PCP, were studied. The removal efficiencies of PCP by immobilized HRP and free HRP were also compared. The stability and

the repeatability is of this method was investigated. The results will provide support for practical application of this method to treat the environmental contaminants, especially in wastewater and wastewater sludge.

2. Materials and methods

2.1. Materials and equipments

HPR (300 U·mg^{-1}) was purchased from Shanghai Xueman Biological Technology Co. Ltd.; PCP (90%, Tokyo Kasei Kogyo Co., Ltd.); Safranin T (95%, Acros Organics); 4-amine methamphetamine (pure chemical, Beijing Xizhong Chemical Factory); Glutaraldehyde (50%, Beijing Yili Fine Chemical Factory); Sodium alginate (pure chemical, Sinopharm Chemical Reagent); Fe_3O_4 (pure chemical, Development Center for Special Chemical Reagents in North China); H_2O_2 (30%), gelatin (pure chemical), isoamyl acetate (analytical grade) and other chemicals were purchased from Beijing Chemical Factory (Beijing, China).

Equipments included a UV757CRT ultraviolet-visible spectrophotometer (Shanghai Precision Scientific Instrument, China), DS-K1 Electric Vibrating Machine (Jinan 2nd Medical Apparatus Factory), SZ-93 water distiller (Shanghai Yarong Biochemical Instrument Factory), and 79-3 magnetic thermostatic stirrer (Radio Components Factory in Shanghai, China).

2.2. HRP immobilization and measurement of enzyme activity

First, a beaker that contained 2.0 g Fe_3O_4 and 2.0 mL of HRP (100 U·mL^{-1}) was incubated at 25℃ for 2 hours. Then, 20 mL of 10% gelatin were added and mixed (v: v=1:10). The mixture was injected onto a culture dish and placed in a refrigerator to condense for 1 hour, the Fe_3O_4 powder adsorbing HRP embedded in the gelatin was obtained. The gel was cut into small pieces (about 3 × 3 × 3 mm). Finally, 40 mL of 0.5% glutaraldehyde were added, and the mixture was cross-linked for 30 minutes. The product was washed six times by deionized water, and re-extracted, sealed at 4℃.

The amount of enzyme decomposing 1 μmol of hydrogen peroxide per minute under the condition (25℃,pH=7.0) is defined as one peroxidase unit (U). For the measurement of enzyme activity, 0.1 mL of HRP (0.4 mg·L^{-1}), 1.4 mL of 4-aminoantipyrine (2.252 mmol·L^{-1}, as indicator), and 1.5 mL of H_2O_2 (2.252 mmol·L^{-1}) was mixed rapidly, transferred to a quartz cell, and scanned at 510 nm in a spectrophotometer, to examine absorbance. The curve that absorbance changed over time was used to calculate the enzyme activity of free HRP, as is calculated by Eq. (1).

$$\text{Enzyme activity per unit } (\mathrm{U/g}) = \frac{\mathrm{E_{510}} \times 3}{6.58 \times \mathrm{E_w}} \times 1000 \qquad (1)$$

Where E_{510} is the increase of absorbance per minute at the wavelength of 510 nm; 3 is the volume of the solution; E_w is the mass of HRP in 0.1 mL of HRP; 6.58 is the constant that the increment of absorbance per minute per U.

For the measurement of immobilized HRP, the formular was applicable. The difference is the E_w indicates the mass of HRP in 1 g of immobilized HRP (wet weight).

2.3. Optimal conditions for PCP removal catalyzed by HRP

A 1.0-mL aliquot of PCP stock solution was diluted to 100 mL with buffer solution, and 2.0 mL of HRP solution was mixed with 50 mL of the PCP solution. In a conical beaker, the H_2O_2 solution was added, and stirred. The concentration of remaining PCP was determined at several time-points. The same method was applicable for free HRP.

Several parameters, including the concentration of free HRP (20.3, 45.5, 71.0, 95.1, 113.7, 136.9 U), glutaraldehyde (0.1%, 0.25%, 0.4%, 0.5%, 0.6%, 0.75%, 1.0%), Fe_3O_4 dosage (0.25, 0.5, 1.0, 1.5, 2.0 g), cross-linking time (10, 30, 40, 60, 120, 180 minutes), gelatin concentration (5%, 10%, 15%, 20%, 30%) were considered by single factor control experiments to determine the optimal reaction conditions.

In the carrier adsorption experiments, de-ionized water was used instead of H_2O_2 to determine the PCP adsorption efficiency. Thus, the difference between the total PCP removal efficiency and the adsorption removal efficiency reflected the rate of PCP removal catalyzed by immobilized HRP.

3. Results and discussions

3.1. Optimal conditions for HRP immobilization

Effects of glutaraldehyde concentration, cross-linking time, Fe_3O_4 dosage, gelatin concentration and free HRP concentration on the enzymatic activity of immobilized HRP were examined. Different concentrations (according to the method in Section 2.2 and 2.3) of glutaraldehyde (40 mL) was added to the mixture of 1.0 g Fe_3O_4 and 120 U HRP. The results showed that when the concentration increased from 0.1% to 0.5%, the enzyme activity saw an obvious increment and peaked at 0.5% (1.48 U/g), and then decreased rapidly with the continuous increasing of glutaraldehyde concentration. So 0.5% was chosen as the optimal glutaraldehyde concentration. As the cross-linking time extended from 10 to 180 minutes, the enzyme activity decreased from 0.94 $U \cdot g^{-1}$ to 0.39 $U \cdot g^{-1}$ gradually. It indicated the more serious intramolecular or intermolecular cross-linking was, the larger the enzyme activity loss was. But by analyzing the relationship between mechanical intensity and cross-linking time, it was found that the immobilized HRP was easily broken after being stirred and shaken for less than 30 minutes. Thus, 30 minutes was chosen as the duration to ensure the application and enzyme activity. With the dosage of Fe_3O_4 increased from 0.25 g to 1.00 g, the enzyme activity increased from 0.71 U/g to the maximum 0.93 U/g, and gradually decreased to 0.54 U/g (2.0 g). That was because the overdose of Fe_3O_4 resulted in the congestion of immobilized HRP molecules and intensified the steric hindrance. Therefore, 1.0 g of Fe_3O_4 was chosen to limit material loss. The difference was insignificant (0.630–0.728 $U \cdot g^{-1}$) when the gelation concentration was at 10 -20 %. The highest enzyme activity (0.88 $U \cdot g^{-1}$) appeared at 5%, but the strength of this gel was low, making it unsuitable for application. In the presence of 30% gelatin, the enzymatic activity of the immobilized HRP was significantly decreased to 0.40 $U \cdot g^{-1}$. The enzyme activity increased from 0.31 U/g to 1.1 U/g with the amount of enzyme rising from 20.3 to 95.1 U, but changed slowly with continuous increase. The recovery rate of enzyme activity reduced to 11.6% from 24.7% gradually with the adding of enzyme amount. It can be explained by the transform from adsorption embedding to gelatin embedding resulting from Fe_3O_4 adsorption saturation.

Above all, the optimal reaction conditions were determined as: the dosage ratio of HRP: Fe_3O_4: gelatin was 95 U: 1.0 g: 10 mL (10–20%); the concentration of glutaraldehyde was 0.5%; the cross-linking time was 30 minutes.

3.2. PCP removal efficiencies by immobilized and free HRP

The PCP removal efficiency changes with time when catalyzed by free HRP and immobilized HRP was shown in Figure 1. The PCP removal process catalyzed by free HRP reached equilibrium within 30 minutes. For the carrier, adsorption was completed after 15 minutes, but the catalytic reaction by immobilized HRP reached equilibrium at 30 minutes. That was because the decreasing of H_2O_2 concentration limited further reaction. In the presence of H_2O_2, the enzymatic activity of HRP lost by 50% within 30 min, leading to the stalling. Compared with the free enzyme, PCP catalytic removal efficiency did not decrease for immobilization.

The effects of pH on catalytic removal of PCP using immobilized HRP in different buffer systems were investigated. The optimal pH range for PCP removal was 4.0 to 6.0. The maximum catalytic removal of PCP (41.0%, 41.3%) was achieved at pH 5.0. Compared with the free HRP, the reaction by immobilized HRP was less influenced by pH, having a broad pH range. The carrier adsorption efficiency in the HAc-NaAc buffer was generally higher than that in the Na_2HPO_4-$C_6H_8O_7$buffer system. The reason may be that the coulomb effects of various salts and specificities of negative ions contributed to the weakening of HRP catalytic activity, although the adsorption effect of the carrier was preserved after immobilization.

The effects of PCP initial concentrations on the removal efficiencies were studied. The results showed that with an increase of initial concentration of PCP from 2.0 mg·L^{-1} to 13.8 mg·L^{-1}, catalytic removal amount of PCP increased from 0.8 mg·L^{-1} to 3.4 mg·L^{-1}, but the catalytic removal rate decreased from 39.7% to 24.4% and the total PCP removal rate decreased from 90.5% to 49.9%. An increase in PCP concentration within a low concentration zone generated more phenoxyl free radicals, which was favorable for the removal reaction. However, a further increase in PCP concentration would result in lower removal efficiency due to hydrogen peroxide depletion. The mechanism of PCP removal by immobilized HRP differs from that achieved by free HRP. This may be because the catalytic ability of HRP was improved after immobilization, and even at low PCP concentrations, its capacity was retained. A proportion of the PCP was adsorbed to the surface of the immobilized HRP, which interfered with the catalytic reaction.

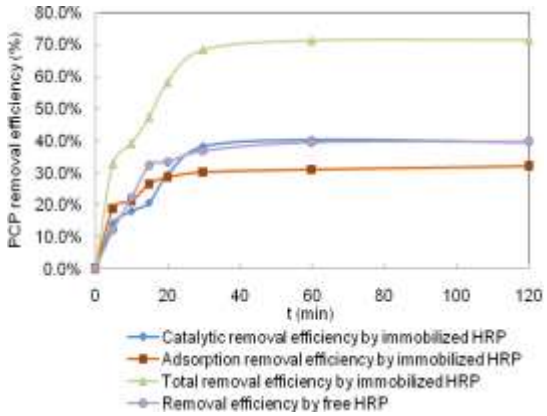

Figure 1: PCP removal rate changes with time catalyzed by free and immobilized HRP.

3.3. Reuseability of immobilized HRP

With the increasing of reuse times, PCP removal rate decreased gradually, while catalytic removal rate remained unchanged or increased gradually. The total PCP removal rate of HRP increased decreased slightly, but the overall are maintained at above 40%. That indicated the good stability and reuseability of immobilized HRP.

4. Conclusions

A simple HRP immobilization method has been developed using Fe_3O_4-adsorption and gelatin embedding. This is a highly effective method to immobilize HRP. The optimum conditions for immobilization are: 0.5% glutaraldehyde; 30 minutes of cross-linking; a ratio of gelatin to Fe_3O_4 of 10 mL (10–15%) to 1.0 g for HRP with Fe_3O_4 95U:1.0 g, which results in an enzyme activity of the immobilized HRP of 1.1 U·g^{-1} (w/w).

The reaction catalyzed by immobilized HRP has a broader pH range and is less influenced by different buffer systems. The initial concentrations of PCP also have effects on the removal efficiency. The immobilized HRP shows stable catalytic activity, which is fit for the processing of waters polluted by PCP at different levels. It requirs less HRP in wastewater treatment compared with free HRP, which had good prospect of practical application. Based on the results, the catalytic reaction device should be developed and application researches need to be carried out.

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