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Degradation of Pentachlorophenol by an Electroenzymatic Method using Immobilized Peroxidase Enzyme

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Abstract−In this study, pentachlorophenol (PCP) was degraded by the electroenzymatic method, which combines the enzymatic catalysis and the electrogeneration of hydrogen peroxide (H_2O_2). The experiments were conducted in a two-compartment packed-bed reactor using horseradish peroxidase (HRP) immobilized electrode. The highest production of H_2O_2 and the current efficiency were observed at –0.4 V vs. Ag/AgCl and a flow rate of 1 mL/min. The highest initial degradation rate and degradation efficiency of PCP were achieved at pH 5 and 25 °C. Under the conditions, the electrolysis was compared with an electrochemical method. The presence of chloride ion indicates that PCP was dechlorinated at the initial period of degradation. According to the proposed breakdown pathway and the intermediates, the electroenzymatic method showed an improved degradation ability compared to an electrochemical method.

Key words: Electroenzymatic Methods, Horseradish Peroxidase, Hydrogen Peroxide, PCP, Degradation

INTRODUCTION

Pentachlorophenol (PCP), one of chlorophenols, has been used for a wood preservative [Micklewright, 1986]. The contamination of PCP in surface and ground water and soil is a major environmental concern [Wu et al., 2002], because PCP is very toxic and persistent to conventional treatment processes such as carbon adsorption and biodegradation. Also these conventional methods have several disadvantages such as the high cost of activated carbon regeneration, waste generation, low degradation rate, and long retention time. In order to overcome these problems and to remove organopollutants efficiently, an electroenzymatic process may be a viable treatment method.

The electroenzymatic method is a process in which an oxidoreductase such as horseradish peroxidase (HRP) is introduced in an electrochemical reactor. Although HRP can degrade a variety of pollutants [Ryu and Kim, 1996], the applications of HRP to processes are rare, because the supply of hydrogen peroxide (H_2O_2), which is necessary to maintain the activity of enzyme, is limited by difficulties in storage and transport of H_2O_2 due to its instability. From this point of view, the combination of HRP immobilized on electrode and an electrochemical cell is an interesting approach. By electrode reactions, H_2O_2 is generated and supplied to the enzyme continuously; consequently, the pollutants can be degraded by enzyme. Based on the concept of the electroenzymatic method, some researches have been reported. Lee et al. studied the degradation of 2,4,6-trinitrotoluene (TNT) by an electroenzymatic method. Previous studies investigated the electrogeneration of H_2O_2 and the degradation of TNT by the electroenzymatic method. Also, the efficiency of electroenzymatic method was higher than that of electrochemical and biochemical methods [Lee et al., 2001a, 2003]. However, the breakdown pathway and the intermediates have not been studied yet. In this study, therefore, the electroenzymatic method was applied to degrade PCP, and the intermediates and breakdown pathway of PCP were investigated. The result of the electroenzymatic method was compared with that of the electrochemical method.

EXPERIMENTAL

1. Materials

Horseradish peroxidase, type VI-A (EC 1.11.1.7), was used. The electrolyte was 0.1 M phosphate buffer (PBS). Ten ppm PCP was added only to the catholyte and the pH was adjusted by using 0.1 N NaOH and H_3PO_4. All the chemicals used in this study were obtained from the Sigma Aldrich Chemical Company (USA) and all solutions were prepared with Milli-Q water. Graphite felt (GF, thickness=6 mm) and reticulated vitreous carbon (RVC, 100 ppi) were obtained from the Electrosynthesis Company (USA).

2. Methods

To increase the stability of HRP, the enzyme was immobilized. The immobilization method used in this study was described previously [Lee et al., 2003]. Electrochemical measurements were performed with a three-electrode system consisting of a platinum electrode, a saturated Ag/AgCl electrode and both the RVC and GF as counter, reference, and working electrodes, respectively. The electrodes were connected to Autolab (263 A EG&G Co.) to measure the voltammograms. The concentrations of H_2O_2 [Kosaka et al., 1998; Lee et al., 2003] and the chloride ion [Zhang and Nicell, 2000] were measured with a UV-visible spectrometer (1601-pc, Shimadzu, Japan) set at 454 nm and at 460 nm, respectively. The concentrations of PCP were measured by HPLC (WATERS™, USA) with the UV detector set at 280 nm. A reverse phase column, Nova Pak®C_{18} (3.9 i.d×150 mm), was used. The mobile phase consisted of 1% acetic acid in methanol and 1% acetic acid in water. The ratio of methanol to water was changed from 40 : 60 to 100 : 0 in 15 min and then changed to 40 : 60 in another 5 min [Kim, 1998]. The flow rate was 1.5 mL/min and the injection volume was 20 μl. GC/MS analyses

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were performed with a 5973N MSD connected to an HP 6890 Network series GC system in order to determine the intermediates and products formed during the reaction. For the analysis, the pH of the sample was adjusted to 3 with HNO_3 and then the sample was extracted twice with diethylether. An HP-5MS column was used for the analysis and the oven temperature was programmed to maintain a constant temperature at 50 °C for 1 min, and then increased to 250 °C at a rate of 20 °C/min, and finally for 10 min at 250 °C. The automatic injection system was kept at 250-280 °C, while the mass detector temperature was held at 280 °C. To identify the products, Wiley library searches were used [Hasio and Nobe, 1993].

3. Electrochemical Cell

The electrochemical cell used in this study was similar to that described previously [Lee et al., 2003] without adding net, placed behind the membrane, to prevent the oxidation of the RVC surface. During the experiments, the working electrode compartment (cathodic compartment) was filled with 100 ppi RVC and GF. The electrolyte moved upward in both compartments. The reactor was operated in a continuous mode (flow rate: 1 mL/min) at –0.4 V (vs. Ag/AgCl) and the catholyte was continuously oxygenated throughout the whole experiment by pumping oxygen at a rate of 25 mL/min.

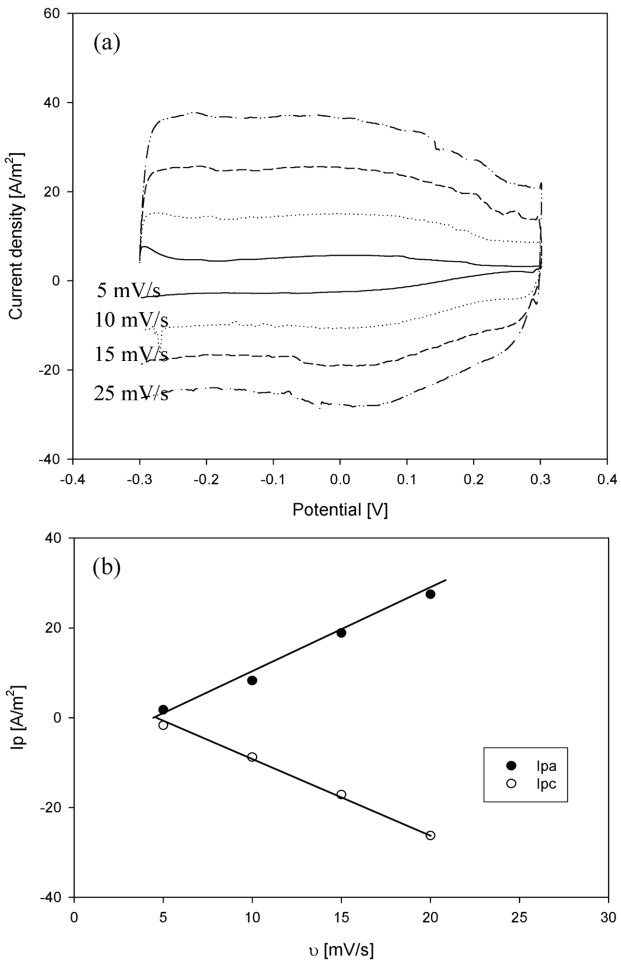


Fig. 1. Cyclic voltammetric response of the HRP immobilized on carbon modified electrode with PCP in 0.1 M PBS (pH 5) at 5, 10, 15, and 20 mV/s (a), Plot of anodic peak and current and cathodic peak current vs. scan rate (b). I_{pa}: anodic peak current, I_{pc}: cathodic peak current, υ: scan rate.

RESULTS AND DISCUSSION

1. Cyclic Voltammetric Studies

Fig. 1(a) shows the voltammograms of a phosphate buffer solution (pH 5) containing PCP with RVC and HRP immobilized on graphite felt electrode. The results at –0.2 V vs. Ag/AgCl show that PCP is oxidized, while those at 0.02 V vs. Ag/AgCl could be ascribed to its reduction. Also, the peak height is proportional to the scan rate. Fig. 1(b) shows the value of the peak current is linearly proportional to the scan rates in the range of 5 to 20 mV/s and the oxidation and reduction peaks have the same height, indicating the occurrence of a reversible electron electrode reaction [Guidelli et al., 2001] within the HRP that is immobilized on carbon electrode used in this electroenzymatic reactor.

2. Electrogeneration of H_2O_2

The rates of H_2O_2 formation can be examined under several operating conditions, such as the voltage, flow rate, and oxygen-sparging rate. In this study, however, the effects of the potential and flow rate on the amount of H_2O_2 generated were determined at an oxygen-sparging rate of 25 mL/min and a temperature of 25 °C.

2-1. Effects of the Voltage

Hydrogen peroxide can be continuously generated by using the two-electron reduction of oxygen on a cathode [Kim, 1998]. To select the proper cathode for generation of H_2O_2, the preliminary voltammetric studies were conducted with either RVC or GF. The voltammograms for both RVC and GF are shown in Fig. 2. There was an obvious increase in the current when RVC was used compared to GF. The rate of H_2O_2 production is proportional to the current intensity when the oxygen supply is not limited [Drogi et al., 2001]. From the linear sweep voltammograms, it was found that RVC was the more suitable cathode for the electrogeneration of H_2O_2, while two successive waves of reduction were observed. The first wave (~–0.3 V) corresponds to the reduction of oxygen and the second wave (~–0.6 V) corresponds to the reduction of H_2O_2 to H_2O. Al-

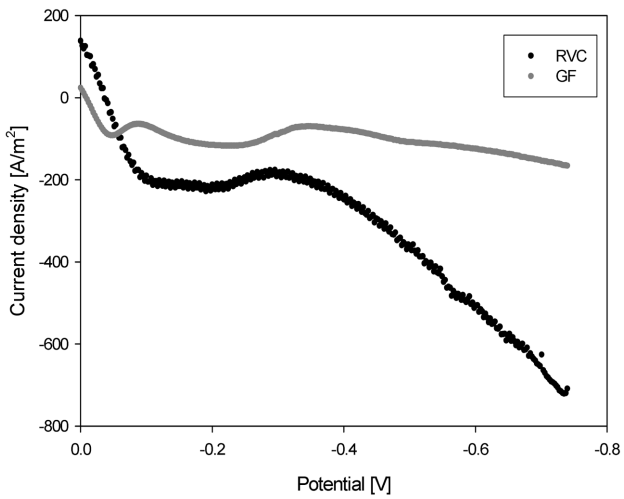


Fig. 2. Linear sweep voltammogram of RVC and GF electrode-sweep rate=20 mV/s, 0.1 M PBS (pH 5), flow rate=1 mL/min, O_2 supply, room temperature.

though RVC is appropriate to generate H_2O_2, but is not for immobilization of enzyme, while the structure of GF is textile suitable for immobilization of enzyme. Therefore, a hybrid of RVC and GF was used to compensate for both the efficient generation of H_2O_2 and the immobilization of enzyme.

To determine the optimum potential for the electrogeneration of H_2O_2, electrolytic experiments were carried out for 60 min with the RVC electrode in the potential range of −0.2 to −0.6 V. During these experiments, the catholyte was 200 mL of 0.1 M PBS (pH 5) saturated with oxygen at a flow rate of 1 mL/min. The concentration of electrogenerated H_2O_2 reached a maximum and remained constant after 40 min (data was now shown). It implies that the H_2O_2 is generated and simultaneously degraded in the system at the same rate [Lee et al., 2003]. The maximum amount of H_2O_2 and the highest current efficiency were both obtained at −0.4 V vs. Ag/AgCl as shown in Fig. 3, and the operating potential for the electro-generation of H_2O_2 was set at −0.4 V for all further experiments.

2-2. Effects of the Flow Rate

The generation of hydrogen peroxide is also influenced by the flow rate. Experiments were carried out with increasing flow rate from 0.5 to 1.5 mL/min. As the flow rate was increased from 0.5 to 1 mL/min, the concentration of H_2O_2 increased but then decreased as the flow rate was further increased (Fig. 4). It may be due to insufficient dissolved oxygen reduction at the cathode-solution interface [Lee et al., 2003]. Therefore, the optimum conditions for the electrogeneration of hydrogen peroxide was set at −0.4 V vs. Ag/

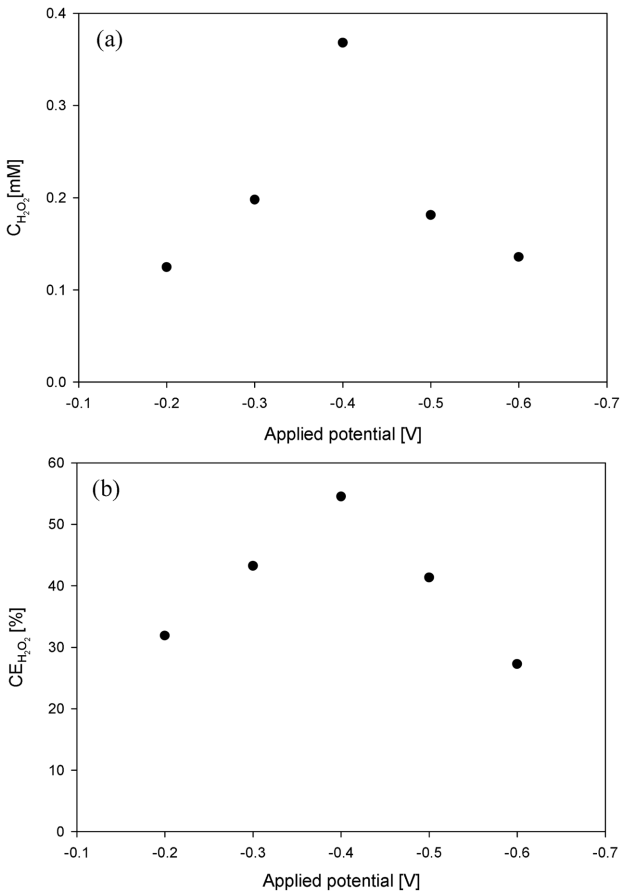


Fig. 3. The effect of potential on the electrogeneration of H_2O_2 for 40 min using RVC electrode, (a) the concentration of H_2O_2 generated and (b) the current efficiency with applied potential: 0.1 M PBS (pH 5), flow rate=1 mL/min, O_2 supply.

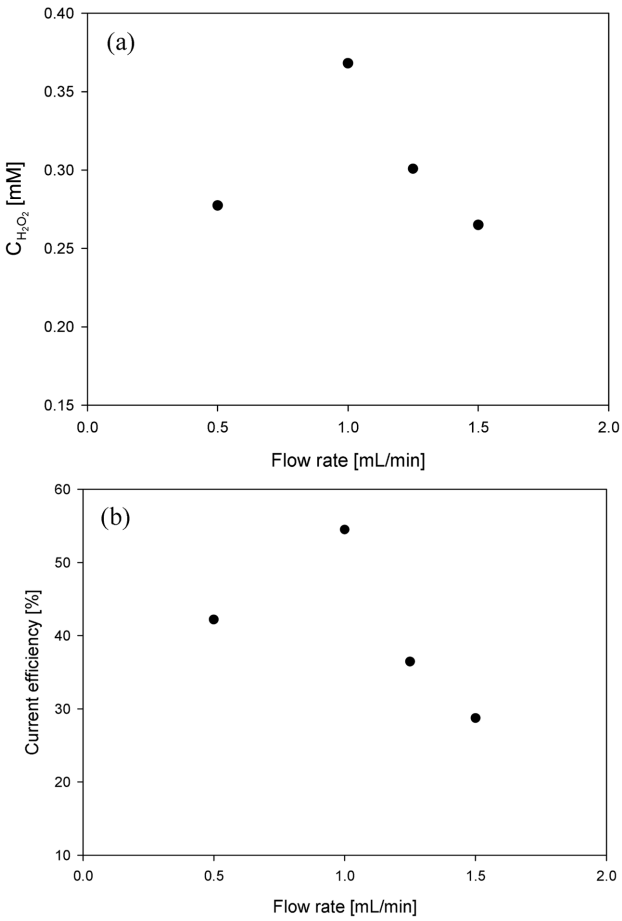


Fig. 4. The effect of flow rate on the electrogeneration of H_2O_2 for 40 min using RVC electrode, (a) the concentration of H_2O_2 generated with the time and (b) the current efficiency: –0.4 V vs. Ag/AgCl, 0.1 M PBS (pH 5), O_2 supply.

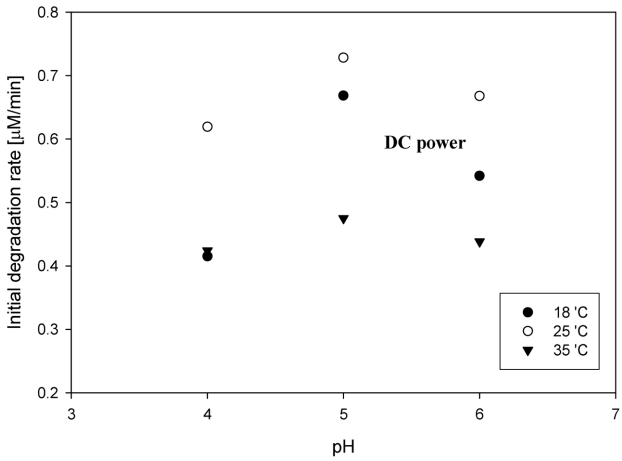


Fig. 5. The effects of pH and temperature on the initial degradation rate of PCP in 0.1 M PBS at 18, 25, and 35 °C: –0.4 V vs. Ag/AgCl, flow rate=1 mL/min, O_2 supply.

AgCl, 1 mL/min of flow rate, 25 mL/min of oxygen supply, and 25 °C.

3. Degradation of PCP

The mechanism for the electroenzymatic reaction is complex and takes place in a number of steps. However, this study is based on the assumptions that the H_2O_2-driven oxidation mechanism is involved in the electrochemical reaction and that the enzymatically inactive form of HRP (Fe^{5+}) does not exist.

3-1. Effects of the pH and Temperature

The effects of pH and temperature on the initial reaction rate were examined in the pH range of 4 to 6 at temperatures of 18, 25, and 35 °C with the same HRP activity. The reactor was operated in a continuous-flow mode. From Fig. 5, it was found that the highest initial reaction rate occurred at pH 5 and 25 °C. The pH value was consistent with the optimum pH obtained in the PCP transformation by the combined action of solution state of HRP and hydrogen peroxide [Zhang and Nicell, 2000]. The degradation efficiency and the initial reaction rate were also determined under the same conditions. Fig. 6 shows the degradation for 100 min. At pH 4 and 5, a high degradation efficiency was observed at 25 °C, but there was no significant difference with the temperature when performed at pH 6. This result may be attributed to the characteristics of the catalytic reaction rate between HRP and H_2O_2. The oxidative reaction, the Fe^{3+} form of HRP is oxidized by H_2O_2, is independent of the pH for values between 4.5 and 7.5. However, the reductive reaction ($Fe^{4+} + R^{\cdot+} \rightarrow Fe^{4+} \rightarrow Fe^{3+}$), is highly dependent upon the pH, showing higher reaction rates in acidic solutions [Nelson and Elisa, 2002]. In this course, a reducing substrate such as PCP was degraded and the degradation efficiency was highly dependent on the enzyme activity. In general the optimum condition for HRP activity was pH 4.5-5.5 and room temperature, which is consistent with the results observed in this study. Therefore, the optimal conditions for the catalytic reaction between HRP and H_2O_2 were determined to be pH 5 and 25 °C.

3-2. The Electroenzymatic and Electrochemical Degradation of PCP

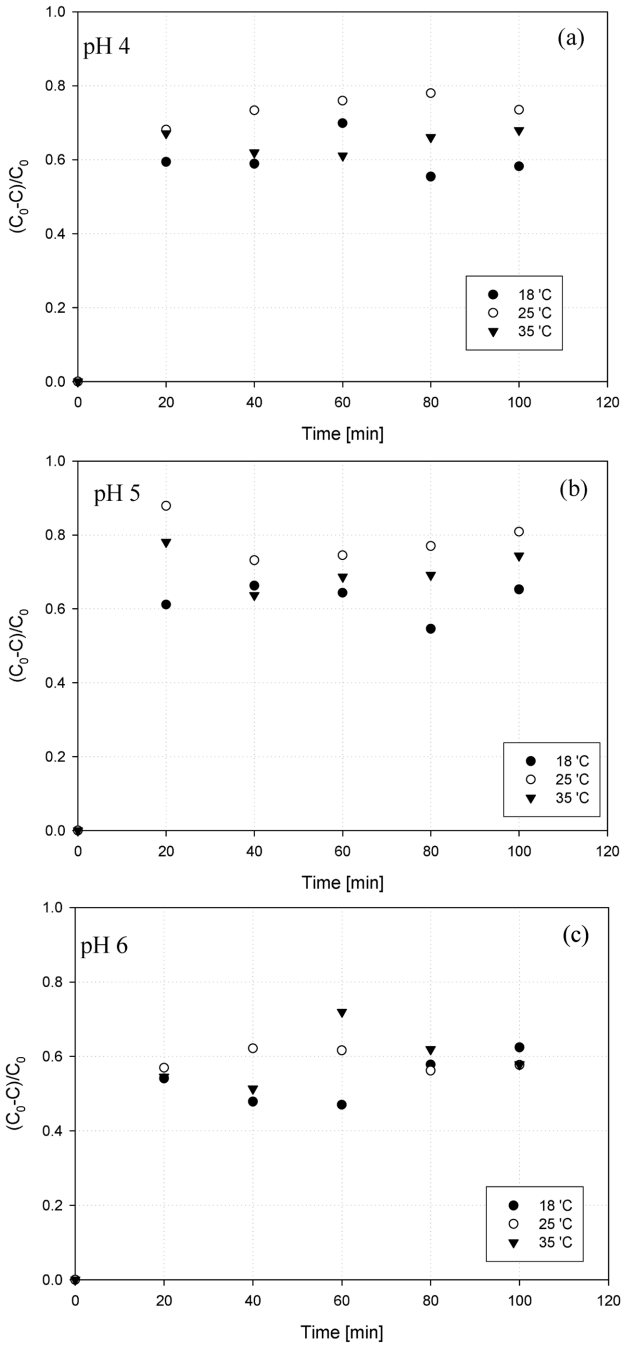


Fig. 6. The effects of temperature on the removal of PCP in 0.1 M PBS (a) pH 4, (b) pH 5, and (c) pH 6: –0.4 V vs. Ag/AgCl, flow rate=1 mL/min, O_2 supply.

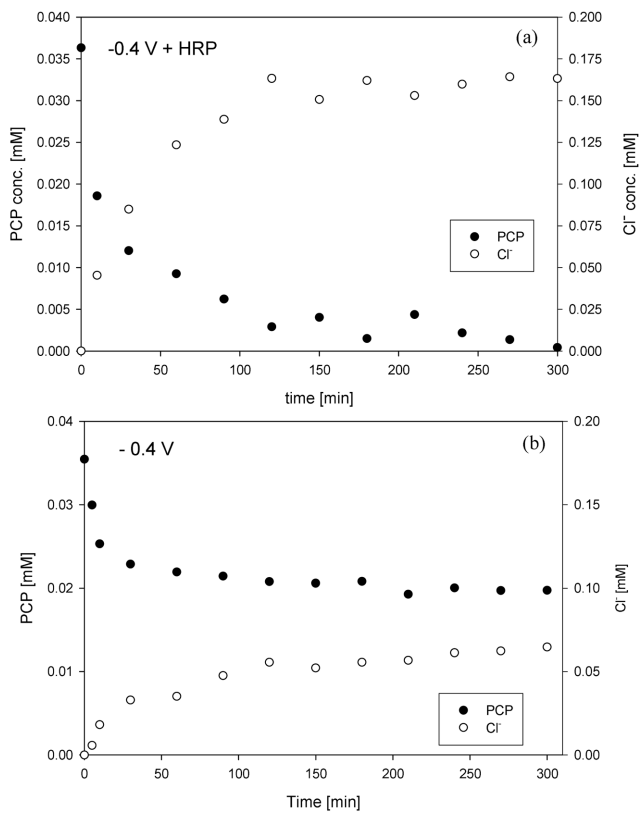


Fig. 7. The degradation of PCP by (a) an electrochemical and electroenzymatic method (–0.4 V+HRP: 0.134 U/mL) and (b) an electrochemical method (–0.4 V): PCP in 0.1 M PBS (pH 5) at 25 °C, flow rate=1 mL/min, O_2 supply=25 mL/min, batch mode.

To compare the electroenzymatic and electrochemical methods for PCP degradation, further experiments were conducted. Under the optimal conditions determined in the above experiments, PCP was degraded in both electroenzymatic and electrochemical methods. Using the electroenzymatic method (−0.4 V+HRP: 0.134 U/mL), the PCP concentration rapidly decreased during the first 30 min period and then decreased at a continuous rate afterwards. As the reaction proceeded, the PCP concentration decreased to approximately 0.1 ppm, while the concentration of chloride ion (Cl^-) was found to be 0.164 mM, based upon the absorbance measurements, indicating that 91% of the initial chloride ions was released from PCP while 1.1% remained in the form of PCP (Fig. 7(a)). When applying the electrochemical method (−0.4 V), although the rate of PCP degradation was high during the first 30 min period, the overall degradation rate was much lower than that of the electroenzymatic method. Even though the reaction time increased further, there was no significant change in either the concentration of PCP or the amount of chloride ion released. The final concentrations for PCP and the Cl^- were 0.02 mM and 0.065 mM, respectively (Fig. 7(b)). More than half (55%) of the total initial chloride ions remained in the form of PCP, while 36% was converted to the ionized form.

Based on these results, the amount of intermediates (such as mono-, di-, tri-, tetra-chlorophenol) would be assumed by the mass balance of Cl^-. One mole of PCP molecule may generate five moles of Cl^-, so the final concentration of Cl^- should be 0.181 mM according to the stoichiometry. From Fig. 8, the mass balance of chloride is the following:

$$[Cl^-]_{total}=[Cl^-]_{pcp}+[Cl^-]_{intermediate}+[Cl^-]_{solution}$$

where, $[Cl^-]_{total}$: the total initial amount of chloride as PCP, $[Cl^-]_{pcp}$: the amount of PCP undegraded as chloride at time t, $[Cl^-]_{intermediate}$: the amount of chloride as intermediates at time t, and $[Cl^-]_{solution}$: the amount of chloride in solution.

For the electroenzymatic method, the total initial amount of Cl^- within undegraded PCP and in solution was 0.002 mM and 0.164 mM, respectively. Therefore, as previously discussed, it was thought that the Cl^- that is unaccounted for, i.e., 0.015 mM (8%), was in the form of intermediates which cannot be quantitatively analyzed. Similarly, for the electrochemical method, it is considered that the remaining chloride ions was 9% of the total initial Cl^- existed in the form of intermediates, which was not detected as Cl^-.

3-3. Breakdown Pathway

In order to identify the breakdown pathway, i.e., the intermediates and reaction products during PCP degradation by the electroenzymatic and electrochemical methods, the samples were analyzed by GC/MS. The degradation of PCP by an electroenzymatic method is a catalytic reaction between HRP and H_2O_2. That is, the Fe(III)

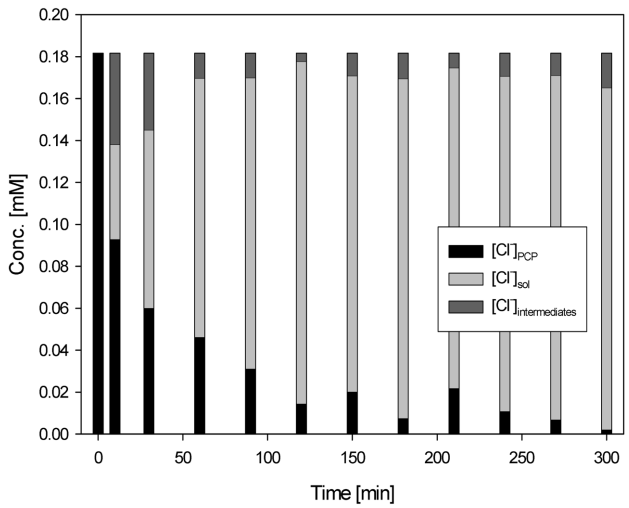


Fig. 8. The mass balance of Cl^- by the electroenzymatic degradation of PCP (–0.4 V+HRP: 0.134 U/mL): PCP in 0.1 M PBS (pH 5) at 25 °C, flow rate=1 mL/min, O_2 supply.

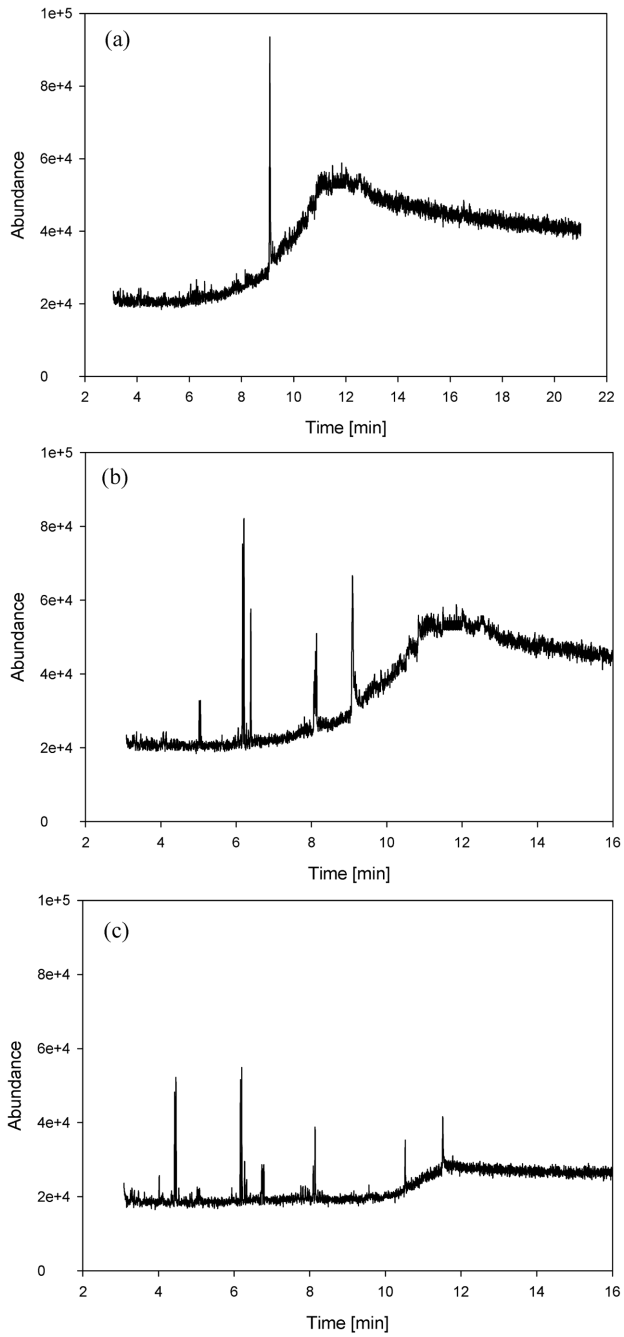


Fig. 9. Total ion chromatograms of PCP solutions, (a): before, (b): during, and (c): after the electroenzymatic degradation.

in HRP was oxidized to Fe(IV)+$R^{\cdot+}$ by H_2O_2, which is generated by the electrode reaction. The oxidized HRP was then reduced to its native form (Fe(IV)+$R^{\cdot+}$→Fe(IV)→Fe(III)) and, at the same time, PCP was oxidized. Therefore, PCP was degraded by HRP in the electroenzymatic process (and/or by H_2O_2 which did not participate in the catalytic reaction with HRP). However, in the electrochemical treatment, PCP was degraded solely by the electrogenerated H_2O_2 and/or by the electrode reaction at electrode surfaces itself.

Total ion chromatograms (TIC) of the PCP, the intermediates and reaction products with GC/MS are shown in Fig. 9, (a) is the initial PCP solution, (b) and (c) are the chromatograms for the samples taken during and those after the electroenzymatic degradation, respectively. Fig. 10 shows the chromatogram (a) during and (b) after the electrochemical degradation. Table 1 shows that the retention times of the chemicals were identified by GC/MS. From the chromatograms, the intermediates were identified and the breakdown pathway of PCP with the electroenzymatic method was proposed as shown in Fig. 11. PCP was initially dechlorinated to form phenol, as indicated by the Cl^- concentration in the sample as detected with a UV-VIS spectrometer. In the course of the conversion of PCP to phenol, it was assumed that intermediates such as 2-,3-, and 4-chlorophenol could exist. In this study, however, the intermediates were not identified. Then methylation took place, phenol was converted

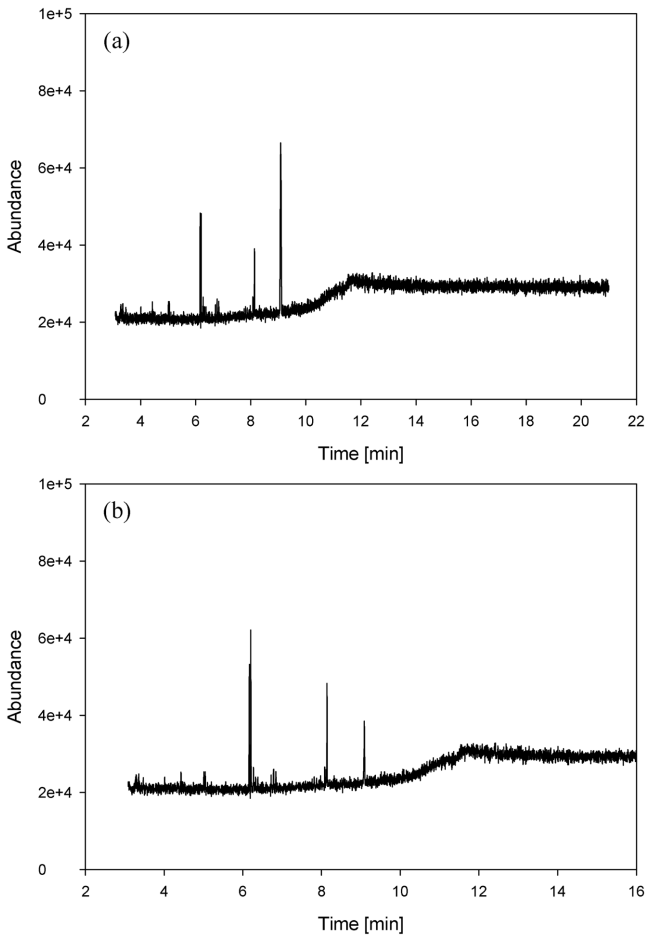
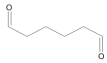
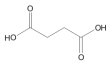


Fig. 10. Total ion chromatograms of PCP solutions, (a): during and (b): after the electrochemical degradation.

Table 1. The retention times of PCP, its intermediates and products analyzed by GC/MS[a]

Name	Structure	Retention time
Undecane		4.43 min
Heptane		4.46 min
Ethanedial		5.05 min
1,3-Di-tert-butylbenzene		6.17 min
1,4-Di-tert-butylbenzene		6.21 min
Hexanedial		6.38 min
Succinic acid		6.79 min
2-Methyl-4,6-bisphenol		8.09 min
4-Methyl-2,6-bisphenol		8.14 min
Pentachlorophenol		9.08 min
Palmitic acid		10.51 min
Stearic acid		11.51 min

to 4-methyl-2,6-bis(1,1-dimethylethyl) phenol and 2-methyl-4,6-bis(1,1-dimethylethyl) phenol or 1,3 or 1,4-di-tert-butylbenzene with the concurrent loss of OH. All these breakdown intermediates generated would undergo further oxidation and ring cleavage to form organic acids, such as succinic acid. Also phenol was partially transformed to aldehydes, such as ethanedial and hexanedial. These short chain linear compounds would then undergo polymerization to form long chain compounds, such as palmitic acids (pentadecanoic acids) and stearic acids (octadecanoic acids) by oxidative C-O and C-C coupling. This reaction is generally seen during the detoxification of phenolic contaminants [Law et al., 2003]. The proposed pathway is similar to that presented by Law et al. and that of photocatalytic oxidation, where both systems are oxidative and utilize hydroxyl radicals to cleave organopollutants [Law et al., 2003; Dec and Bollag, 1990; Guillén et al., 2000].

The intermediates and products of the electrochemical reaction were also analyzed. According to the GC/MS results, it was found that the PCP degradation intermediates and reaction products with the electrochemical method (by electrogenerated hydrogen peroxide) were different from those of the electroenzymatic method. Fig. 12 shows a PCP breakdown pathway with the electrochemical method. Similar to the electroenzymatic method, the PCP initially went through the dechlorination step, then degraded by the course of methylation, losing -OH, ring cleavage, and finally transformed to succinic acid. However, no aldehydes and long chain compounds were seen in the electroenzymatic method. Therefore, the electroenzy-

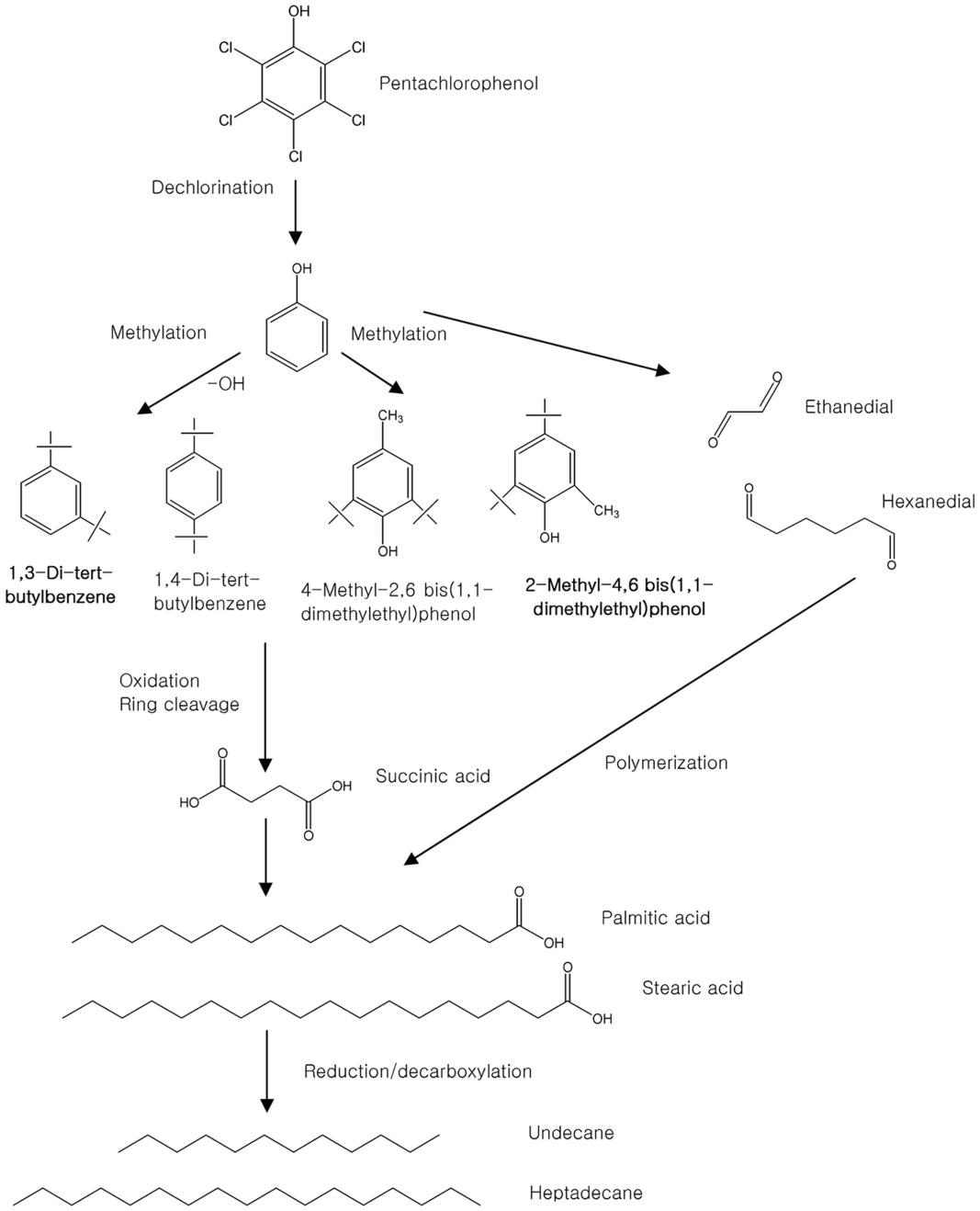


Fig. 11. The proposed breakdown pathway of PCP by electroenzymatic method.

matic method shows more enhanced degradability than the electrochemical method based upon the final products. Also, there was the possibility of reactions between the intermediates which were generated in the breakdown pathway of PCP, but the reaction and reaction products were not further identified in this study.

CONCLUSION

In this study, the electroenzymatic method was applied to degrade PCP by using immobilized HRP and electrogenerated H_2O_2. Using the HRP immobilized on carbon electrode, we examined the cyclic voltammograms of PCP depending on the scan range and the scan rate, and it was confirmed that a reversible reaction occurred. The optimum condition for the electrogeneration of H_2O_2 was investigated. Under the optimum condition, the highest degradation efficiency of PCP was obtained at pH 5 and 25 °C. To compare the electroenzymatic and the electrochemical methods for the degradation of PCP, the intermediates and reaction products of each method were examined in addition to the degradation efficiency. From the results, a higher degradation efficiency was obtained by using the electroenzymatic method, and it was confirmed that PCP went through the dechlorination step first from the presence of the chloride ion in the electrolyte. According to the proposed breakdown pathway and the intermediates, the electroenzymatic method showed

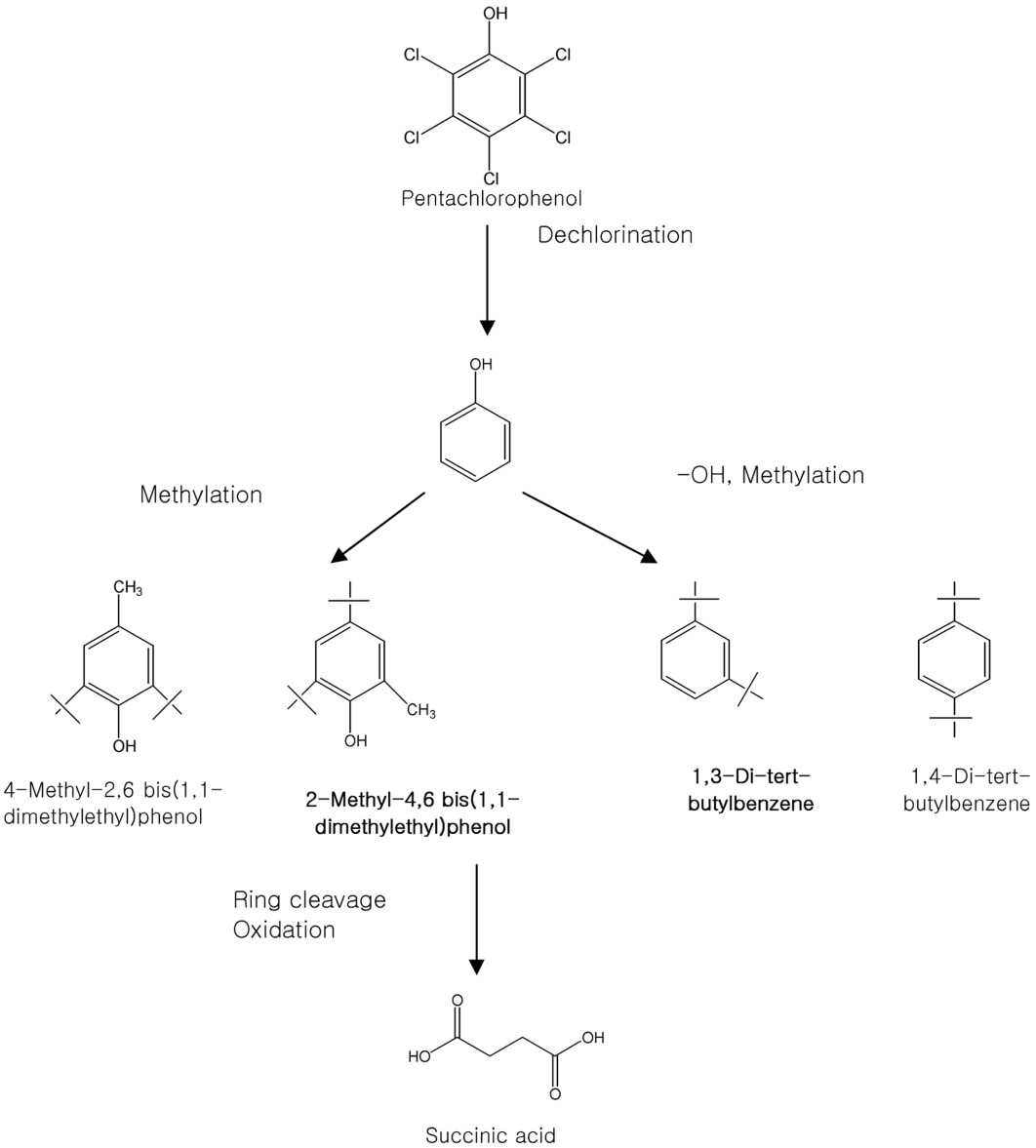


Fig. 12. The proposed breakdown pathway of PCP by electrochemical method.

more improved degradation ability than an electrochemical method. Therefore, an enzyme-based electrolytic treatment process might be a viable approach for the removal of PCP and the other persistent organic pollutants.

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