Korean J. Chem. Eng., **24**(1), 72-78 (2007)

Degradation of azo dye by an electroenzymatic method using horseradish peroxidase immobilized on porous support

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(*Received 21 February 2006 • accepted 30 July 2006*)

Abstract−An electroenzymatic process is an interesting approach that combines enzyme catalysis and electrode reactions. Degradation of orange II by an electroenzymatic method using horseradish peroxidase (HRP) bound on inexpensive and stable inorganic beads was studied in a continuous electrochemical reactor with *in situ* generation of hydrogen peroxide. HRP was immobilized on Celite®R-646 as a porous support with 2% aqueous glutaraldehyde (GA), while the protein and activity yield were 3.6 mg protein and 5,280 U per g Celite, respectively. Based on a parametric study, the operating conditions were chosen, and over 90% of the degradation efficiency of orange II was maintained during continuous operation for 36 hr. From the results of GC/MS analysis, degradation products were identified and a possible breakdown pathway of orange II was also proposed. This study shows the feasibility of an electroenzymatic process to degrade azo dye compounds in wastewater.

Key words: Electroenzymatic Method, Horseradish Peroxidase, Electrogeneration of Hydrogen Peroxide, Covalent Binding

INTRODUCTION

Multitudes of synthetic dyes are used for textile dyeing, paper printing, and color photography, and more than 50% of these are azo dyes at a discharge amount of 7×10^5 tons/year [Kim et al., 2005]. Their release into the environment by way of industrial effluence is a major concern because reduction of azo linkages may generate potentially toxic, carcinogenic or mutagenic aromatic amine compounds [Brown et al., 1981; Vaidya and Datye, 1982; Brown and Devito, 1993; Chivukula et al., 1995; Daneshvar et al., 2003; Na et al., 2004]. Azo dyes are usually resistant to aerobic degradation during conventional biological processes [Chivukula et al., 1995; Zhang et al., 2005], but they are easily reduced by reductases such as cytochrome P-450 [Huang et al., 1979; Kulla et al., 1983; Brown and DeVito, 1993] and white-rot fungus [Paszczynski et al., 1992; Spadaro et al., 1992]. They can also be degraded by various advanced oxidation methods such as photocatalytic oxidation, Fenton's reaction, wet air oxidation, zero-valent iron reduction and other advanced oxidation processes [Sparado et al., 1994; Cao et al., 1999; Nam and Tratnyek, 2000; Bauer et al., 2001; Choi et al., 2004; Zhang et al., 2005]. These methods, however, have some drawbacks when used in industrial wastewater treatments, including batch systems, long contact time and low removal efficiency. Compared to these methods, the electroenzymatic process appears to be a promising alternative for degrading organopollutants.

Previous studies revealed that the electroenzymatic process, which is a combined system of enzymatic catalysis and electrode reaction, would be potentially of value in treatment of a variety of organopollutants such as phenol, chlorophenol, nitrotoluene, and azo dye [Lee et al., 2001, 2002, 2003; Kim and Moon, 2005; Kim et al., 2005], and that the most of operating conditions were determined by hydrogen peroxide (H_2O_2) generation for the activation of enzyme and the degradation of pollutants. In these studies, graphite felt and reticulated vitreous carbon (RVC) were used for enzyme immobilization. However, the porous carbon support materials have limitations in practical application due to their high cost and low chemical stability. Therefore, in order to increase the applicability of an electroenzymatic process to an actual system, Celite, an inorganic support having microporous structure, was employed for the immobilized enzyme beads to be used in an electrochemical reactor for degradation of orange II, an azo dye in this study. Furthermore, instead of the periodate linkage, glutaraldehyde (GA) was introduced as a cross-linker to achieve long term stability of the immobilized enzyme and further to utilize the enzyme more effectively [Vasudevan and Li, 1995; Zheng and Xiao, 2004]. In addition, the optimal conditions for the HRP immobilization, the electrogeneration of H_2O_2, and the removal efficiency of orange II were discussed.

EXPERIMENTAL

1. Materials

Horseradish peroxidase, type IV-A (1,280 U/mg, EC 1.11.1.7), and Celite beads (Celite® R-646, 8/14 mesh) were purchased from Sigma and Celite Corporation, respectively. Other chemicals used in this study were obtained from Sigma Aldrich Chemical Company (USA) and all solutions were prepared with distilled water.

2. Preparation of Aminopropyl Celite (APC) and HRP Immobilization

Fig. 1 illustrates the derivation and immobilization steps. The surface of Celite beads has hydroxyl groups (-OH) that provide a mildly reactive surface for activation and enzyme binding. Aminopropyl Celite (APC) can be derived using 3-aminopropyltriethoxysilane with the hydroxyl groups on the surface of Celite to modify the organic functional group ($-NH_2$, aliphatic amine). HRP is covalently

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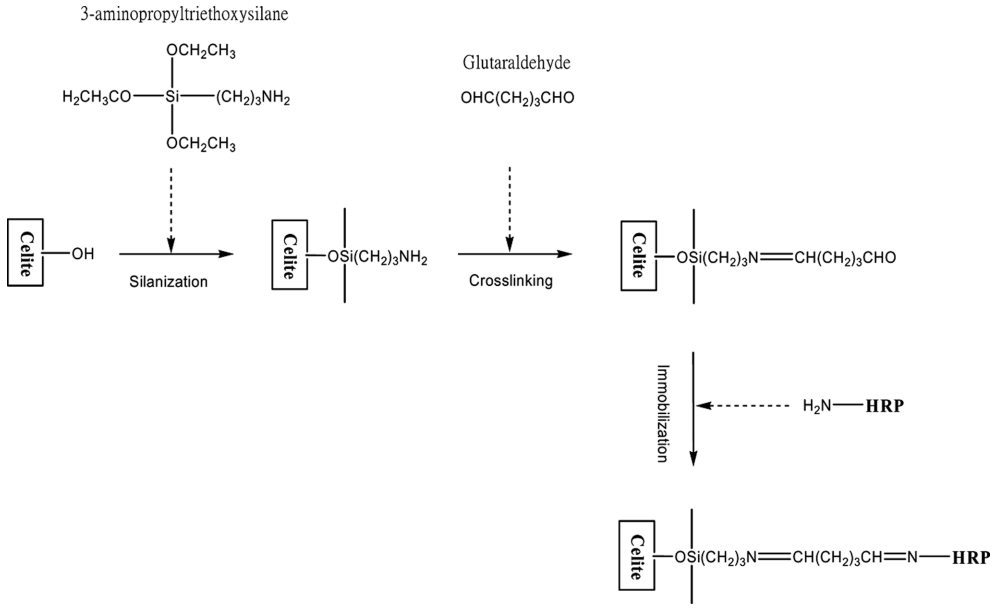

Fig. 1. Synthesis of aminopropyl-celite (APC) and immobilization of horseradish peroxidase onto the APC by glutaraldehyde (GA) linkage.

immobilized onto the APC by using glutaraldehye (GA). GA forms a Schiff's base (aldimine) linkage between carbonyl groups (-CHO) and free amino groups on the HRP, especially those from lysine residues. GA binds proteins through their free amino groups, especially those from lysine residues [Cabral and Kennedy, 1991].

The Celite beads were extensively washed by stirring for 1 hr in distilled water, and a 10% (v/v) aqueous solution of 3-aminopropyltriethoxysilane adjusted to pH 4 with 6 N HCl was added. After incubation at 70 °C for 3 hr with occasional mixing, the liquid was decanted and the beads were dried at 110 °C overnight. The synthesized APC beads were then thoroughly washed with a large volume of distilled-deionized water and dried at 80 °C overnight [Janolino and Swaisgood, 1982]. The APC beads were immersed in 2% (v/v) aqueous GA solution for 2 hr at room temperature. The GA concentration around the Celite beads was uniform due to constant agitation. The GA linked beads were then thoroughly washed with distilled-deionized water and immersed in a known strength of HRP solution (2,000 U/mL) at room temperature (23 °C) for 5 hr and then at 4 °C for 19 hr with constant agitation. The HRP immobilized Celite beads were thoroughly washed with distilled-water and ready for use [Vasudevan and Li, 1995].

3. Design and Operation of an Electroenzymatic Reactor

Fig. 2 shows the experimental set-up used in this study. A two-compartment circular membrane reactor was used and a cation exchange membrane (Nafion 117, DuPont, USA) was placed between the two compartments. Two circular plates (d=5 cm) of platinum-coated titanium were used as the working electrode (cathode) and counter electrode (anode). The HRP immobilized Celite beads were packed into the cathodic compartment. The anolyte was 100 mM phosphate buffer solution (PBS, pH 7.0), and certain concentrations of orange II (azo dye) in the same PBS were used for the catholyte. The anolyte was circulated in the anodic compartment, and the catholyte was continuously fed by two variable speed peristaltic pumps (Masterflex®, U.S.A.). Oxygen gas (50 mL O_2/min) was supplied into the vessel of catholyte for the saturation of dissolved oxygen.

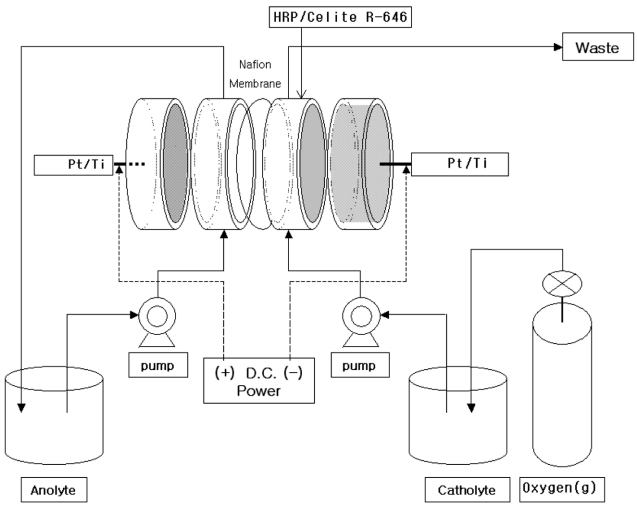

Fig. 2. Schematic diagram of an electroenzymatic reactor.

The experiments were conducted under a constant current mode (15 A/m^2) using a DC power supply (6613C, Agilent, USA).

4. Analytical Methods

Soluble and immobilized HRP enzymatic activities were spectrophotometrically determined by using H_2O_2 as oxidizing substrate and 2,2'-azino-bis-(3-ethyl-benzthiazoline-6-sulfonate) (ABTS) as reducing substrate [Pina et al, 2001; Cardosi, 1997]. The method for determination of protein concentration using bicinchoninic acid was followed in the manner suggested by Smith et al. [1985]. A Fourier transform infrared spectrometer (FTIR, 460 Plus, Jasco, Japan) was used to confirm the functional groups of the original, aminopropyl and GA linked Celite beads during HRP immobilization procedure presented in Fig. 1. The samples were dried at 70 °C, and ground with potassium bromide powder, and finally pressed at 55 MPa to form uniform pellets. Analyses of orange II and hydrogen peroxide were done by using the calibration method. The residual

concentration of orange II was determined at λ_{max}=484 nm by UV-VIS spectrophotometer (UV-1601PC, Shimadzu Co.) [Kim et al., 2005], and the concentration of H_2O_2 was measured at 453 nm according to a colorimetric method using copper (II) ion and 2,9-dimethyl-1,10-phenanthroline [Kosaka, 1998]. Chemical oxygen demand (COD) was measured by a colorimetric method (Standard Methods - 5220 C). Total organic carbon (TOC) was measured with a carbon analyzer (Seivers-820, SEIVERS, CO) connected to an auto sampler (Seivers-820A, SEIVERS, CO). TOC measurements are based on the oxidation of organic compounds to form carbon dioxide using UV radiation and a chemical oxidant (ammonium persulfate). A gas chromatography/mass spectrometry (GC/MS) system was used to identify degradation products in the electroenzymatic reaction.

The samples for GC/MS analysis were pretreated based on the following method. Each sample (20 mL) was acidified by using HCl (pH<2.0). The acidified sample was extracted with dichloromethane (80 mL). The extracts were dehydrated with anhydrous sodium sulfate overnight. The dehydrated sample was evaporated to concentrate (1 mL), and used for GC/MS analysis. An HP 6980 Network series GC system equipped with an HP-5MS capillary column (30 m×0.25 mm×1.0 μm film thickness) and interfaced directly to a 5973N MSD was used as a detector. The oven temperature was programmed: initial temperature of 40 °C held for 10 min, and linearly increased to 100 °C at a 12 °C/min rate, then to 200 °C with 5 °C/min, and finally ramp up to 270 °C with a 20 °C/min, and held at 270 °C for 10 min. Wiley Library Searches were used to identify dye degradation products.

RESULTS AND DISCUSSION

1. Preparation of HRP Immobilized Celite Beads

The HRP immobilized Celite beads were prepared by using GA as a cross-linker. While GA enhances enzyme immobilization, a certain level of GA may inhibit the proteinous activities. To investigate the effects of cross-linking agent and to determine its optimal content of GA, HRP immobilized APC beads were prepared with different GA concentrations. The effect of the GA concentration on the protein yield is presented in Fig. 3. The optimal concentration of GA was determined as 2% (v/v) due to inhibition of enzyme activity by a higher concentration of GA. In previous researches on enzyme immobilization using GA, most of experiments were carried out at room temperature. We found that enzyme activity was significantly influenced by the preparation temperature. In this study, the immobilized HRP was prepared by a step change in temperature. As shown in Fig. 4, the HRP activity as well as protein yield increased after the temperature was changed and finally 3.6 mg protein/g Celite and 5,280 Units/g Celite were obtained. In addition, the storage stability of the immobilized HRP was measured in comparison with free HRP. As shown in Fig. 5, the activity of free HRP was maintained at about 90% of the initial activity for 20 days but sharply decreased to 50% in 40 days. However, the activity of the immobilized HRP remained stable for a longer time and maintained over 90% of its initial activity up to 60 days. These results indicate that the immobilized HRP is more stable than the free HRP because the immobilization procedure may provide a better environment for enzyme activity [Shuler and Kargi, 1992], which is one of the important factors to be considered in the electroenzymatic

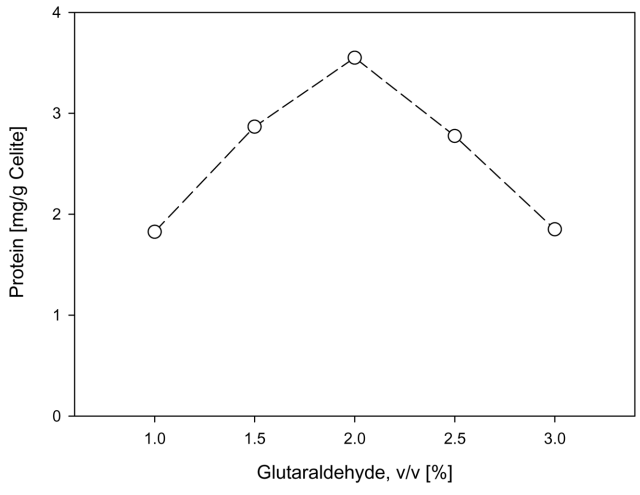

Fig. 3. The effect of GA contents on the amount of protein linked.

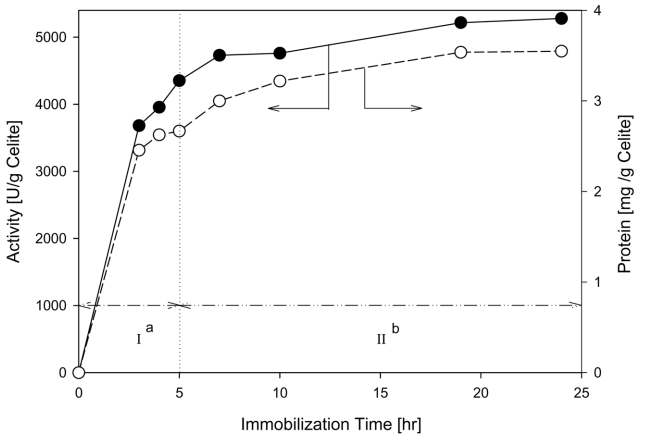

Fig. 4. The effect of temperature and time on the HRP activity (●) and protein yield (○) (a: Step I at room temperature (25 °C), b: Step II at 4 °C).

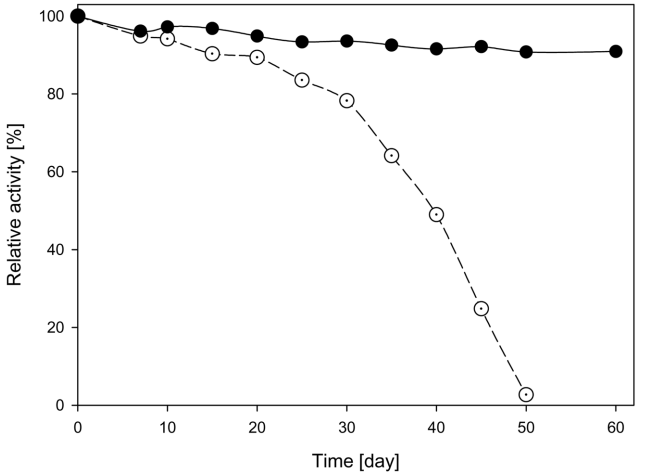

Fig. 5. Storage stability of free and immobilized HRP at 4 °C in distilled-deionized water, (⊙): free HRP, (●): immobilized HRP.

reactor.

2. Electrogeneration of H_2O_2

The HRP activity is activated by the hydrogen peroxide (H_2O_2) and, consequently, the efficiency of the electroenzymatic process is dependent on the generation of H_2O_2. In order to determine the optimal conditions, the effects of current density, retention time, and concentration of buffer solution on the generation of H_2O_2 were examined. Using a DC power supply, the applied current density was varied in the range of 5 to 30 A/m^2. As shown in Fig. 6, the highest concentration of H_2O_2 electrogenerated was observed at 15 A/m^2. Beyond that point, the amount of H_2O_2 decreased and the energy consumption increased significantly. There are two kinds of O_2 reduction, (i) $O_2+4H^++4e^-\leftrightarrow 2H_2O$ (E^0=1.229 V vs. NHE) and (ii) $O_2+2H^++2e^-\leftrightarrow H_2O_2$ (E^0=0.695 V vs. NHE) [Bard and Faulkner, 2000]. Actually, a higher potential is needed to reduce O_2 to water [Lee et al., 2003]. Therefore, it was assumed that a voltage at a higher current density than 15 A/m^2 may exceed the electrode potential to form H_2O_2 electrochemically. In further experiments, the current density was fixed at 15 A/m^2 for the electrogeneration of H_2O_2.

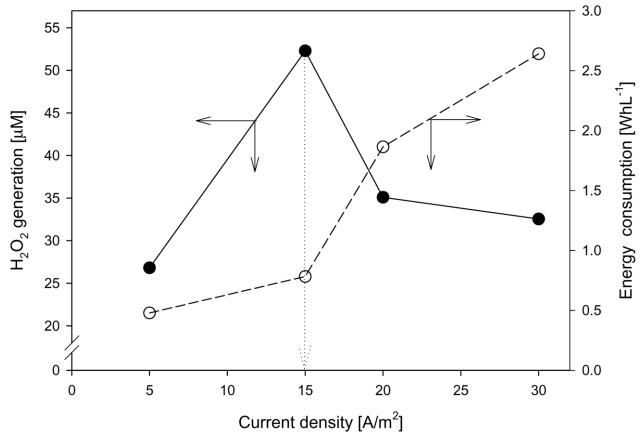

Fig. 6. The Effect of current density on the generation of H_2O_2 (●) and the energy consumption (○). [PBS]=100 mM, retention time=45 min and temp.=25 °C.

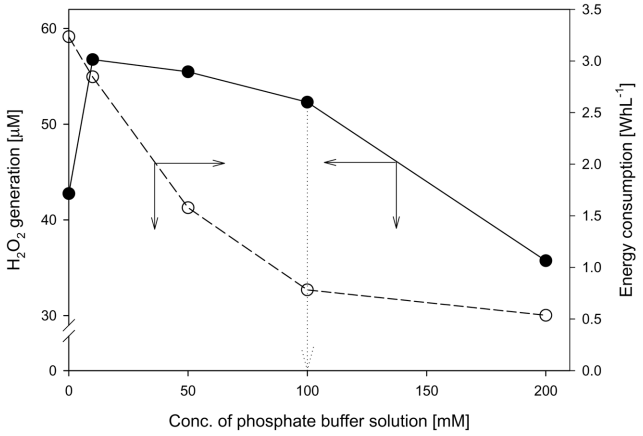

Fig. 7. The effect of concentration of buffer solution on the generation of H_2O_2 (●) and the energy consumption (○). Current density=15 A/m^2, retention time=45 min and temp.=25 °C.

The effect of concentration of the buffer solution (catholyte) was also investigated in the range of 0 to 200 mM (pH 7) at 15 A/m^2. As shown in Fig. 7, the highest H_2O_2 concentration was observed at 10 mM of PBS, but in this region, the energy consumption was markedly high. Considering H_2O_2 concentration as well as energy consumption, a reasonable concentration of PBS was determined as 100 mM. In addition, the effect of retention time on the electrogeneration of H_2O_2 was investigated. As shown in Fig. 8, the concentration of H_2O_2 increased with increasing retention time due to its accumulation, while the lowest energy consumption was observed at 45 min or a longer of retention time. But it is thought that a shorter retention time is advantageous for reactor operation. Based on the results, the experimental conditions were set at 15 A/m^2 current density, 100 mM phosphate buffer solution and 45 min retention time for further experiments.

3. Effects of pH and Temperature on Enzymatic Degradation of Orange II

It was reported that the variations in pH of the medium affect the ionic form of the active site of enzyme and change the activity and the three-dimensional structure of the enzyme [Shuler and Kargi,

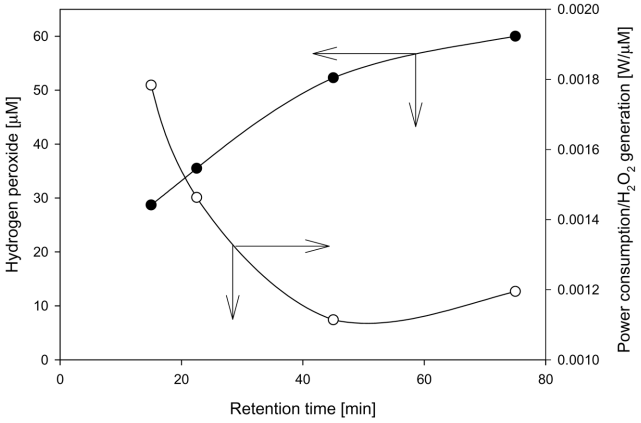

Fig. 8. The effect of retention time on the generation of H_2O_2 (●) and the power consumption (○). Current density=15 A/m^2, [PBS]=100 mM and temp.=25 °C.

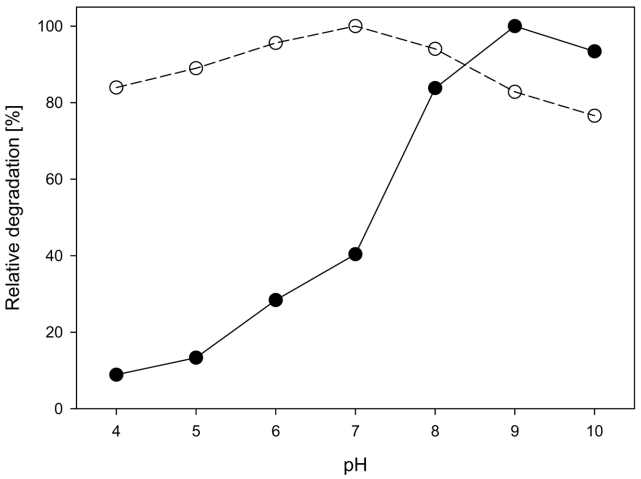

Fig. 9. The effect of pH on the removal efficiency of orange II by free (●) and immobilized (○) HRP.

1992]. The experiments were carried out in the range of pH 4 to 10 to investigate the effects of pH on the removal of orange II. Either 100 μL of HRP solution (1,000 U/mL) or 1 g of HRP immobilized Celite beads (5,280 U/g) was added to a batch system (50 mL) at 25 °C with 50 mg/L of orange II solution in PBS (100 mM) at each pH condition, and 100 uM of H_2O_2 was externally injected. Fig. 9 shows the removal efficiencies of orange II depending on pH in the free and the immobilized HRP system. The highest removal efficiency was obtained at pH 7 and 9 for immobilized and free HRP, respectively. Furthermore, the removal efficiency for the free HRP system significantly varied with pH, while that for immobilized HRP system was relatively stable and higher than that of the free enzyme. This implies that the immobilized HRP has a higher stability against the pH variation owing to the protection of enzyme by immobilization.

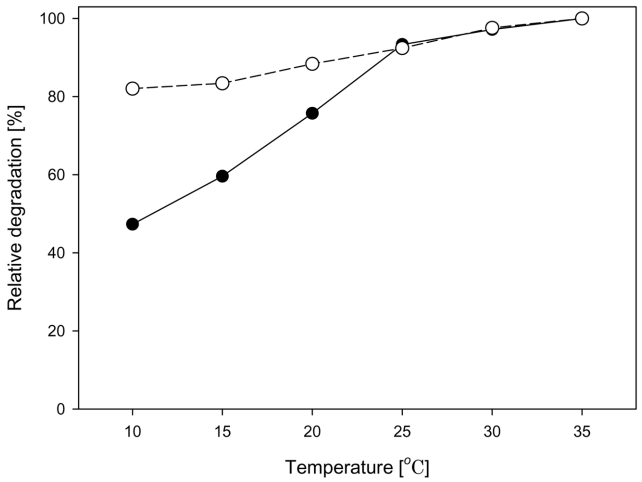

Fig. 10. The effect of temperature on the removal efficiency of orange II by free (●) and immobilized (○) HRP.

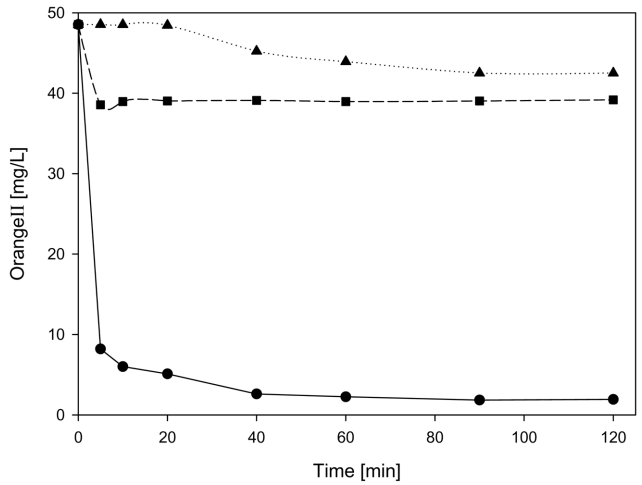

Fig. 11. Removal of orange II by electroenzymatic (●), electrochemical (▲) and adsorption method (■), electroenzymatic method in the presence of HRP immobilized Celite beads under 15 A/m^2, electrochemical method under 15 A/m^2, adsorption method in the presence of HRP immobilized Celite beads [Orange II]$_o$=50 mg/L in PBS (100 mM, pH 7), retention time=45 min and temp.=25 °C.

The rate of enzyme-catalyzed reactions increases with temperature up to a certain limit, but above a certain temperature, enzyme activity decreases due to denaturation [Shuler and Kargi, 1992]. To examine the effect of temperature on the removal efficiency and to determine the optimal condition, the removal of orange II was conducted in a temperature range of 10 to 35 °C (operational temperature). As shown in Fig. 10, the removal efficiency increased for free and immobilized enzymes with increasing temperature up to 35 °C. However, at a lower temperature, relatively higher removal efficiency was observed for the immobilized HRP system. The results imply that immobilization protected the enzyme activity from variation of temperature also.

4. Operation of the Electroenzymatic Reactor

In order to confirm the effectiveness of the electroenzymatic degradation, electroenzymatic, electrochemical, and adsorption methods were compared. A constant current density (15 A/m^2) and HRP immobilized Celite beads were employed for the electroenzymatic method. As shown in Fig. 11, orange II was not well degraded without an applied electric field, due to the absence of hydrogen peroxide, even in the presence of the enzyme. Applying a current of 15 A/m^2, yet degradation of orange II was insignificant due to the absence of HRP. The electroenzymatic method shows over 90% degradation of orange II, demonstrating the efficacy of the process.

UV/VIS spectra obtained from orange II solutions with different reaction time during electroenzymatic degradation are shown in Fig. 12. The absorption spectrum was characterized by two peaks in the ultraviolet region at 230 and 310 nm and two peaks were observed in the visible region at 405 and 484 nm. The absorbance peaks at 230 and 310 nm are due to the benzene and naphthalene rings of orange II, respectively, while the two peaks at 405 and 484 nm arise from the azo bond [Stylidi et al., 2003]. As shown in Fig. 12, absorption peaks at 230, 310, 405 and decreased at 484 nm, but a new peak was observed at 205 nm with the increasing reaction time. These results are caused by the breakdown of the azo bond and formation of the aromatic amines from orange II. When orange II is degraded, the azo bond (-N=N-) is cleaved, hence the absorbance at 484 nm

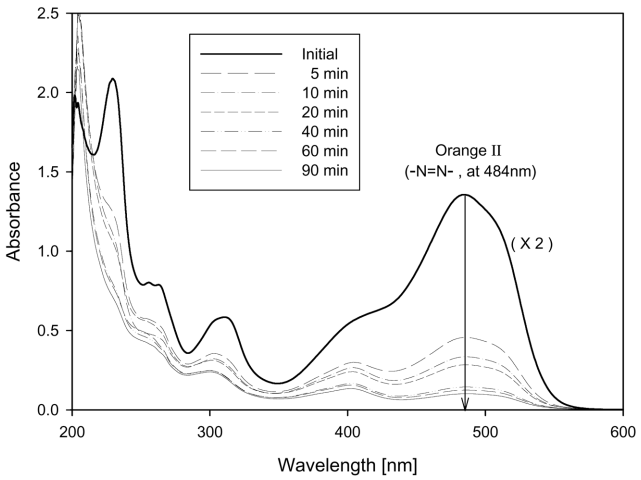

Fig. 12. UV-VIS spectral changes of orange II in the electroenzymatic process as a function of time [Orange II]$_o$=50 mg/L, pH=7 and temp.=25 °C. Initial sample was diluted two times before measurement, and the others were not.

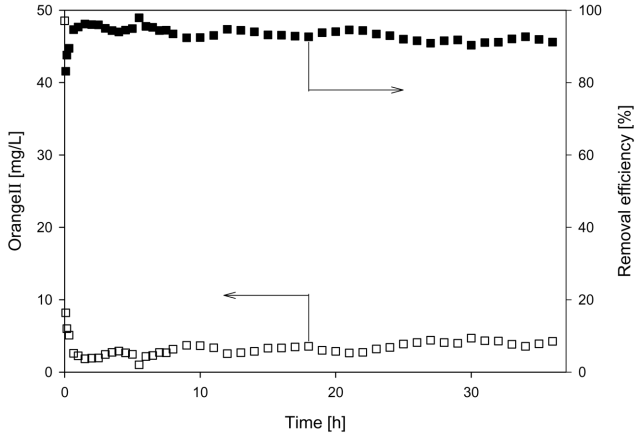

Fig. 13. The change of residual concentration (□) and removal efficiency of orange II (■) by electroenzymatic process for 36 hr [Orange II]$_o$=50 mg/L in 100 mM PBS (pH 7), current=15 A/m^2, retention time=45 min and temp.=25 °C.

reduces [Kim et al., 2005]. As further reactions proceeded, substituted aromatic amines were generated and shifts in the characteristic wavelengths were observed.

To investigate the stability of the electroenzymatic process, a continuous reactor, a two-compartment circular reactor was operated for 36 h under a constant current density (15 A/m^2). This test was performed using 50 mg/L of orange II in PBS (100 mM, pH 7) at 25 °C. Fig. 13 shows the change in residual concentration and removal efficiency of orange II as a function of time. The concentration of orange II immediately decreased from 50 mg/L to less than 5 mg/L during the operation. The removal efficiency of orange II was over 90% for 36 h.

5. Degradation Products of Orange II

In order to identify the breakdown species of orange II by the electroenzymatic process, the samples were analyzed by GC/MS. As shown in Fig. 14, several alkenes, such as 1-docene, 2-dodecene, 3-tetradecene, 1-hexadecene, 1-octadecene, 1-nonadecene, 5-eicosene and 1-docosene were detected and these products are relatively well-degraded compounds. Therefore, it can be concluded that orange II was degraded and changed to easily degraded products by the electroenzymatic process.

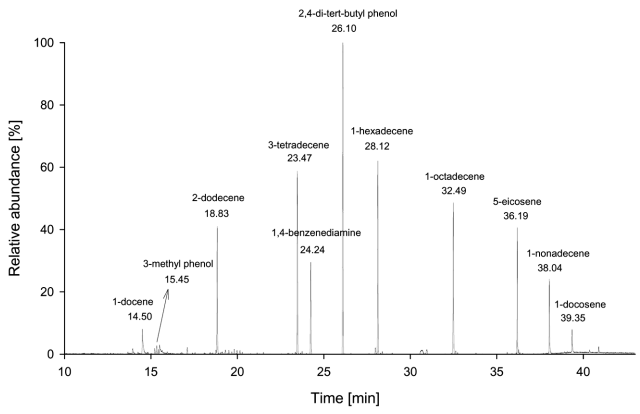

Fig. 14. GC chromatogram of orange II after the treatment of electroenzymatic process.

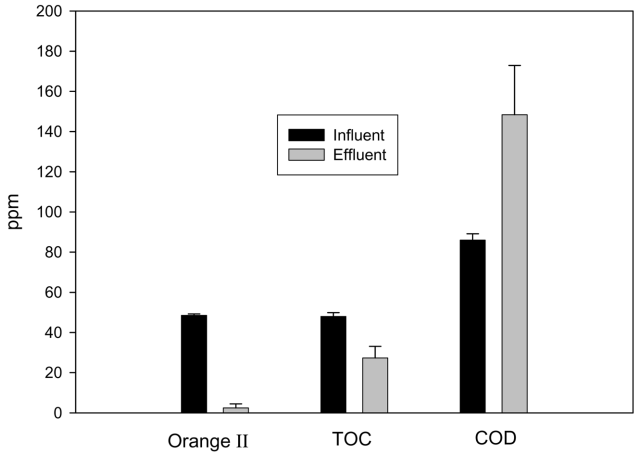

Fig. 15. The changes of the concentration, TOC and COD value of orange II by the electroenzymatic process. Error bars represent the standard error of the mean of three independent measurements.

To examine the mineralization of orange II, total organic carbon (TOC) and chemical oxygen demand (COD) were measured. Fig. 15 shows the changes in concentration, TOC and COD values before and after treatment of the electroenzymatic process. The removal efficiency of TOC was below 45%, whereas that based on the concentration of orange II was over 90%. This indicates that no significant mineralization occurred and a substantial portion of degradation products exists as organic compounds. Interestingly, the COD value increased markedly after the treatment. The COD is used as a measure of the oxygen equivalent of the organic matter content of a sample susceptible to oxidation by a strong chemical oxidant (potassium dichromate). Actually, the electroenzymtic removal of orange II was conducted in the cathodic compartment, which has reduction environment. The reduction environment might induce polymerization of the degradation products and produce several long chain alkenes. For these reasons, the COD value increase dafter the electroenzymatic treatment.

CONCLUSION

The electroenzymatic process is a notable approach in degrading organopollutants by using enzyme and electrode reactions. This study was focused on utilization of an inexpensive and chemically stable support material for HRP immobilization. The enzyme immobilization method was also modified and the HRP immobilized Celite led to high stability and activity. Also, using the immobilized HRP, parametric studies for degradation of orange II were performed. The results show that a low-cost microporous inorganic support can be used in a practical application of the electroenzymatic method with improved process efficiency.

ACKNOWLEDGMENT

This work was funded by Korea Institute of Environmental Science and Technology (KIEST, ECO Project number: 071-031-071).

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