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Electroenzymatic degradation of azo dye using an immobilized peroxidase enzyme

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Abstract

Azo dyes are largely resistant to biodegradation and persist in conventional wastewater treatment processes. Combining enzymatic catalysis and the electrochemical generation of hydrogen peroxide (H_2O_2), an electroenzymatic process was developed, which is a potential alternative to traditional processes. In this study, an electroenzymatic method that uses an immobilized horseradish peroxidase enzyme (HRP), was investigated to degrade orange II (azo dye) within a two-compartment packed-bed flow reactor. To evaluate the electroenzymatic degradation of orange II, electrolytic experiments were carried out with 0.42 U/mL HRP at −0.5 V. It was found that removal of orange II was partly due to its adsorption to the graphite felt. The overall application of the electroenzymatic led to a greater degradation rate than the use of electrolysis alone. Also the by-products formed were found to consist primarily of an aromatic amine, sulfanilic acid, and unknown compounds.

Keywords: Azo dye; Electroenzymatic method; Horseradish peroxidase; Hydrogen peroxide

1. Introduction

Azo dyes are widely used in textile and dyestuff industries. They are produced in quantities that exceed 7×10^5 tons/year [1]. There is considerable environmental interest in color removal in a wide range of wastewaters. Traditional techniques for treating dye wastewater are biological treatment, adsorption on activated carbon and chemical oxidation. Although these technologies are effective and employ relatively simple equipment, they have several drawbacks, such as high operational costs and limited applicability. Also, biological treatments are relatively ineffective in effluent decolorization, because high molecular weight compounds, such as dyes, are not easily degraded by bacteria and, thus, they pass through the treatment system largely undegraded [2]. Some of the disadvantages of conventional treatment methods can be overcome by the use of an enzyme-based reactor, since enzymes catalyze a reaction over a broad concentration range [3] and require a low retention time of the substrate in the reactor.

Specifically, the use of plant peroxidases in removal of phenolic pollutants from aqueous solution is well documented [4,5]. For example, it has been demonstrated that horseradish peroxidase (HRP) can catalyze free-radical formation, followed by spontaneous polymerization of a variety of aromatic compounds, including phenol [6–9], chlorophenols [10,11], and other substituted phenols [12], in the presence of hydrogen peroxide (H_2O_2). Further, HRP is known to efficiently cleave aromatic azo compounds in the presence of H_2O_2 and to degrade and precipitate industrially important azo dyes [13–15]. The previous studies showed that the various phenolic and azo compounds were degraded by HRP–H_2O_2 system. Also it was proved that the immobilized HRP is better than the free HRP in terms of removal efficiency [15]. These studies were conducted with an external supply of H_2O_2 for maintenance of the activity of HRP in degrading the pollutants. However, the use of H_2O_2 is limited partially

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because of its instability and difficulties in storing and transporting the oxidants. Therefore, development of an efficient and stable method for the continuous generation of H_2O_2 was pursued. In this sense, electrogeneration is an attractive approach since it does not require additional chemicals, and electricity is readily available.

Electroenzymatic process is an interesting approach to efficiently utilize H_2O_2-dependent enzymes in biocatalysis [16–20] since it combines the enzymatic catalysis and the electrochemical generation of the H_2O_2 in such a way that overcomes the major limitation, i.e., supply of unstable oxidant. In an anodic oxidation process, the water molecules are adsorbed on the anode surface and then oxidized by the anodic electron transfer reaction. In an indirect oxidation process at the cathode, a strong oxidant, H_2O_2, is generated by electrochemical reactions and used for HRP immobilized on the carbon electrode, thereby accelerating the degradation of chemical pollutants present in the aqueous solution. In the previous studies, the persistent pollutants, such as trinitrotoluene (TNT) and pentachlorophenol (PCP) were degraded more efficiently by the electroenzymatic process than other biochemical and electrochemical methods [16–20]. Specifically, in the electroenzymatic process, the less mole number of H_2O_2 was consumed to degrade 1 mol of TNT than the biochemical process [19]. Also it was found that more advanced reaction proceeded indicated by intermediates and degradation products. These studies demonstrated the feasibility in enzymatic removal of toxic organics from synthetic wastewaters.

The objective of this study is to prove the applicability of the electroenzymatic degradation of azo dyes. Prior to degradation experiments, cyclic voltammetry tests were carried out to examine the reduction-oxidation reactions of an azo dye, orange II, which was chosen as a model compound as it is a representative of the large class of dyes used commercially. Electrolytic degradation experiments were carried out under the optimum potential for H_2O_2 electrogeneration while differences between the electrochemical and electroenzymatic degradation of the dye were investigated using graphite felt alone and a HRP-immobilized graphite felt at the same potential. The other detailed experimental procedures were described previously [18].

2. Materials and methods

2.1. Materials

Horseradish peroxidase, type VI-A (EC 1.11.1.7), was purchased from Sigma. The electrolytes were 0.1 M phosphate buffer (PBS, pH 5.5) and 0.03 mM orange II (azo dye) was added only to the catholyte. 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 (thickness = 6 mm) was obtained from Electrosynthesis Company (USA).

2.2. Immobilization method

Graphite felt (2 cm × 5 cm) was air-oxidized at 500 °C for 8 h, immersed in 1 M hydrazine aqueous solution for three days, and washed with Milli-Q water. After adding 0.1 mL sodium periodate (0.1 M) to a 1 mL solution (0.05 M acetate buffer–0.1 M sodium chloride, pH 5) of HRP (2 mg, 987 U/mg), the partially oxidized HRP was added to the pretreated graphite felt. The graphite felt, with the immobilized HRP, was washed thoroughly with 0.1 M PBS prior to use. The immobilization method used in this study was described elsewhere [18,19].

2.3. Two-compartment packed-bed reactor

A two-compartment packed-bed flow reactor was used and a cation exchange membrane (Nafion 450, Aldrich, USA) was placed between the two compartments to allow proton transport between the compartments. A net was placed adjacent to the membrane to prevent the oxidation of graphite felt's surface. Two plates (2 cm × 5 cm) of platinum-coated titanium were used as a counter electrode (anode) and a graphite current collector. The graphite felt, with the immobilized HRP (HRP: 0.42 U/mL), and an Ag/AgCl electrode were used as a working electrode (cathode) and a reference electrode, respectively. During the experiments, the working electrode compartment (cathodic compartment) was filled with the HRP-immobilized graphite felt. The electrolyte flowed upward in both compartments. The reactor was operated in a continuous mode (flow rate: 1 mL/min) at −0.5 V (versus Ag/AgCl) and the catholyte was continuously oxygenated throughout the whole experiment by pumping 10 mL oxygen/min. Fig. 1 shows the two-compartment packed-bed reactor used in this study.

2.4. Analysis methods

Samples were taken from the reactor effluent for analysis. The concentrations of H_2O_2 [21] and orange II were measured in an UV–vis spectrometer (1601-pc, Shimadzu, Japan) at 454 and 483 nm, respectively. The UV–vis spectra of the samples were recorded by scanning from 190 to 500 nm to check for the formation of intermediates and the decolorization of orange II. The reaction products were measured by HPLC (Waters, USA) using a C-18 column (Nova Pak, 3.9 mm × 150 mm i.d.) and absorbance at 254 nm. The mobile phase gradient was initiated with a 1 mL/min of water/methanol (95:5, v/v) and the ratio of methanol was then increased at the rate of 3%/min.

2.5. Cyclic voltammetry

Electrochemical measurements were performed with a three-electrode system comprising a platinum wire as an auxiliary electrode, a saturated Ag/AgCl electrode as reference against all potentials, and the graphite felt as a working

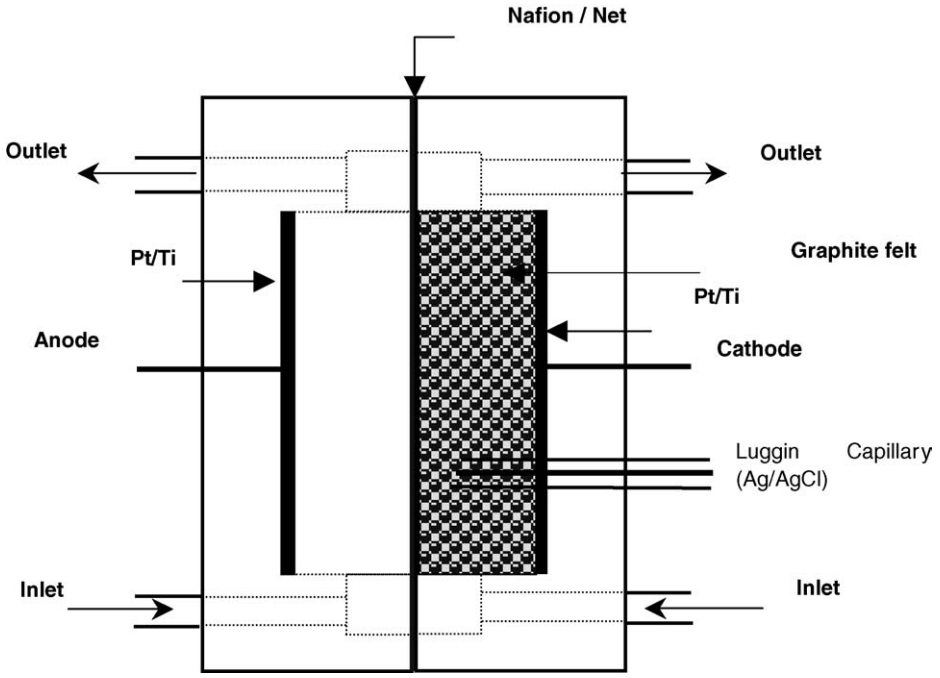

Fig. 1. Schematic diagram of the two compartment packed bed flow reactor.

electrode. The electrodes were connected to Autolab (263 A EG&G Co.).

3. Results and discussion

3.1. Cyclic voltammetry studies

Cyclic voltammetry (CV), one of the most commonly used electroanalytical techniques, has main advantage of ability to characterize an electrochemical system. Fig. 2 shows the voltammograms of orange II in 0.1 M PBS along with the working electrode at the scan rate of 1, 5, 10, and 20 mV/s. In general, the cyclic voltammetry was measured without flow after removing O_2, because O_2 is oxidized and reduced easily, making it difficult to identify the redox peaks of a chemical species under the presence of O_2. However, in this study the voltammograms were obtained under the condition of flowing electrolyte and sparging O_2, to generate H_2O_2 which enables the electroenzymatic process to work efficiently. Therefore, the electrolyte flow and sparged O_2 led to broad peaks by disturbing the diffusion boundary layer on the electrode surface. The results at −250 mV versus Ag/AgCl show that orange II is reduced, while those at 150 mV can be ascribed to its oxidation. The peak current heights were linearly correlated with a square root of scan rate (data was not shown) indicating diffusion phenomena near the working electrode.

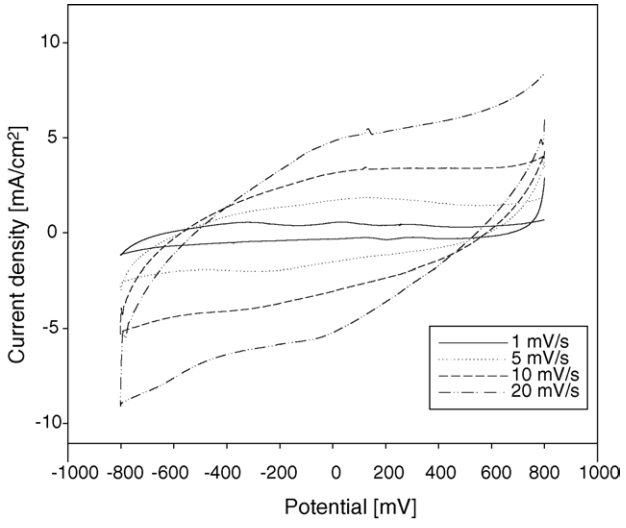

Fig. 2. Cyclic voltammetric response of the HRP-immobilized graphite felt electrode with orange II in 0.1 mol/L phosphate buffer (pH 5.5) at 1, 5, 10, and 20 mV/s.

3.2. Electrogeneration of H_2O_2

In order to find the optimum potential for H_2O_2 generation, electrolytic experiments were carried out. Earlier it was observed that the degree of H_2O_2 formation was approximately constant after 5 min and there was no effect on the H_2O_2 formation [19]. To determine the optimum potential for H_2O_2 generation, the cyclic voltammograms were measured using 0.1 M PBS and a bare graphite felt. From Fig. 3, the first wave (∼−300 mV) corresponds to the reduction of oxygen to hydrogen peroxide and the second wave (∼−700 mV) corresponds to the reduction of hydrogen peroxide to water. Therefore, the electrolytic experiments were carried out for 20 min at potentials ranging from −300 to −700 mV. During these experiments, the catholyte was 200 mL of 0.1 M PBS

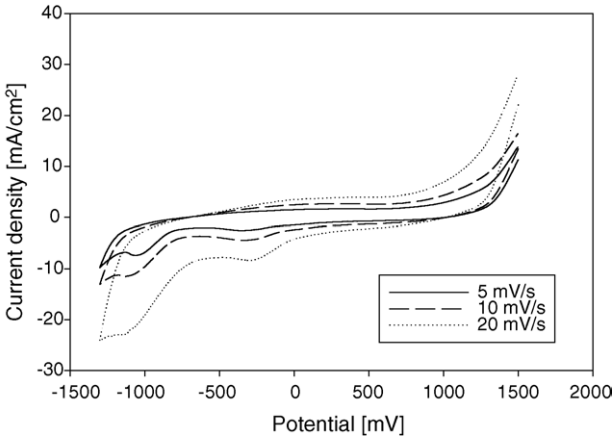

Fig. 3. Cyclic voltammogram of the graphite felt electrode with 0.1 mol/L phosphate buffer (pH 5.5) at 5, 10, 20, and 50 mV/s.

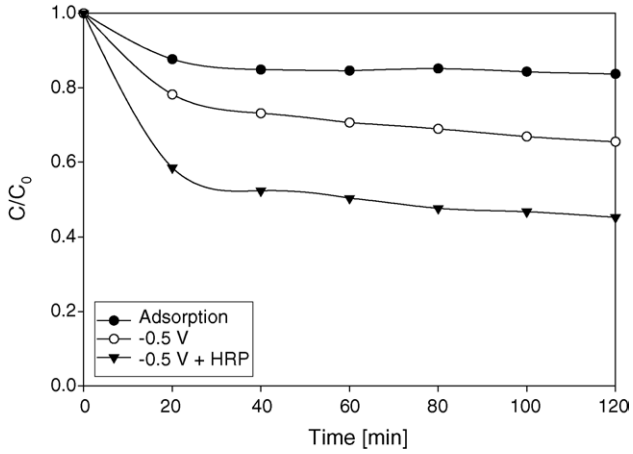

Fig. 4. Removal of orange II with and without the electrogeneration of hydrogen peroxide at −0.5 V both with and without HRP immobilization: 0.03 mM orange II, 0.42 U/ml HRP, flow rate: 1 mL/min, electrolyte: 0.1 M phosphate buffer (pH 5.5), oxygen sparging rate: 10 mL/min.

(pH 5.5) saturated with oxygen. The overall current efficiency for H_2O_2 generation ($CE_{H_2O_2}$) was calculated from changes in the concentration and the electrical charge involved using Eq. (1), where $C_{H_2O_2}$ is the concentration of hydrogen peroxide (mol/L), V is the volume of the electrolyte (L), F is the Faraday constant (96,485 C/mol), and Q is the quantity of the accumulated current, in coulombs [8].

$$CE_{H_2O_2} = \frac{2FC_{H_2O_2}V}{Q} \times 100\% \quad (1)$$

Table 1 shows that the maximum amount of H_2O_2 was generated at −0.6 V, and the highest current efficiency for the electro-generation of H_2O_2 was obtained at −0.5 V. However, there was no significant difference in the amount of H_2O_2 generated between −0.5 and −0.6 V and, therefore, the operating potential for the electro-generation of H_2O_2 was set at −0.5 V for all further experiments. Also it was assumed that inactivation of HRP did not occur. When excess of H_2O_2, the active site, Fe^{3+} of HRP is changed to Fe^{5+}, this state of HRP being called compound III, the activity of which is lost. However, it was reported that the relative activity of HRP started to decrease when the concentration of H_2O_2 is above than 1 mM [22]. Here, as shown in Table 1, the concentration of H_2O_2 electrogenerated was lower than 1 mM. Therefore, it was thought that HRP activity was maintained at −0.5 V.

Table 1
Concentration of H_2O_2 and the current efficiency as a function of applied voltage

Applied potential (V)	Concentration of H_2O_2 (mM)	Current efficiency (%)
−0.3	0.138	31.08
−0.4	0.120	30.88
−0.5	0.227	63.24
−0.6	0.243	16.92
−0.7	0.209	11.73

Parameters used in numerical analysis.

3.3. Degradation products of orange II

Experiments for removal of orange II were carried out using two different methods—electrochemical and electroenzymatic methods. At −0.5 V, graphite felt alone and the HRP-immobilized graphite felt were employed in the electrochemical and the electroenzymatic method, respectively. Fig. 4 shows that the concentration of orange II in both systems decreased with the reaction time. In order to examine the effects of adsorption on the removal of azo dye, the degradation rates were compared with the adsorption experiment. These results indicate that the removal of orange II was affected partly by its adsorption to the graphite felt. A control experiment was conducted without electric current in order to investigate the effect of adsorption. It was found that approximately 13% of orange II was adsorbed.

Since it was found that orange II adsorbed to the graphite felt, graphite felt was initially saturated with orange II followed by electrochemical and electroenzymatic degradation of orange II. Using the HRP immobilized on the graphite felt, orange II was degraded by the electroenzymatic mechanism. The sample at each reaction time was scanned with UV–vis spectrometer. The maximum visible absorption wavelength of orange II was observed at 483 nm but it has a characteristic absorption wavelength in the UV range also [7]. Fig. 5 shows the absorbance at 483 nm decreased from 0.887 to 0.218 during the electroenzymatic degradation of orange II. The results show that there were changes at other characteristic wavelengths. The absorbance at 191 nm increased and the peak at 228 nm shift to 247 nm as the reaction time increased. This change resulted from the breaking down of the azo bond and formation of the aromatic amines from orange II. When orange II was degraded, the azo double bond was severed, and the absorbance at 483 nm was reduced. As the further reaction proceeded, substituted aromatic amines were generated and shifts in the characteristic wavelengths were observed. The

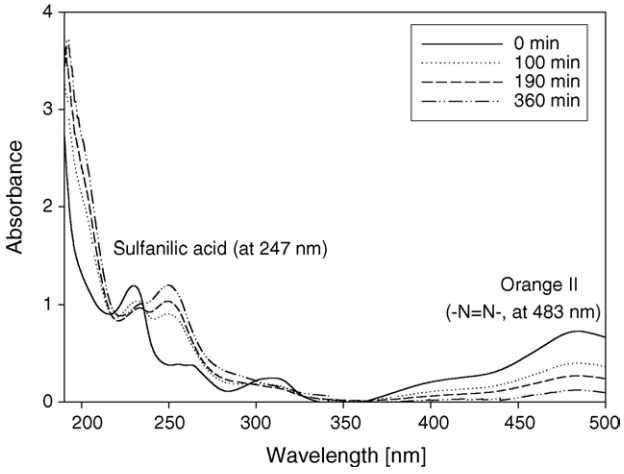

Fig. 5. UV–vis absorption spectrum showing the degradation of orange II for different reaction times.

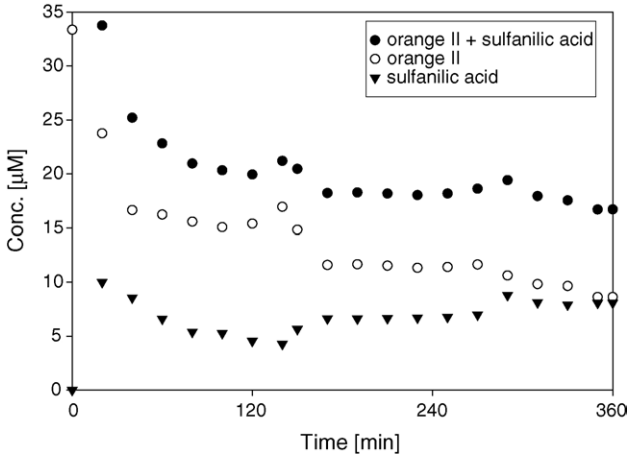

Fig. 6. Time-dependent mass balance of orange II and sulfanilic acid using the optimized electroenzymatic degradation protocol.

results of this study show that the degradation of orange II produces sulfanilic acid, the appearance of which is consistent with reductive cleavage of the azo group. Another by-product is presumed to be 1-amino-2-naphthol. These products were very similar to those from the treatment of orange II by AOP (advanced oxidation process) and photocatalytic process [23,24]. However, it was not possible to identify further species because the other intermediates might be generated by the autoxidation of 1-amino-2-naphthol [25]. These products, sulfanilic acid and 1-amino-2-naphthol were found during the electrochemical degradation also. The appearance of sulfanilic acid indicates that orange II was reduced at the cathode. Since the cathodic compartment was packed with the HRP-immobilized graphite felt, and graphite felt is a porous carbon electrode, it has a large surface area and large capability to adsorb compounds. Therefore, orange II would be absorbed to the felt, where it was reduced to sulfanilic acid.

3.4. Degradation of orange II by electroenzymatic and electrochemical method

In order to quantify the presence and the concentration of sulfanilic acid, orange II, and other degradation products, the reaction sample was analyzed by HPLC. Fig. 6 shows the time course for orange II degradation, with the HRP-immobilized graphite felt, and the concurrent appearance of sulfanilic acid. During the first operation, 54% of the orange II was degraded within 120 min. Subsequent runs found that 66 and 74% of the orange II was degraded during the second and third operations, respectively. While a total of 55% was degraded after the third run when using the electrochemical method (data was not shown). Seventy four percent of orange II was degraded during the electroenzymatic experiment as shown in Fig. 7. When 1 mol of orange II is degraded theoretically, 1 mol of sulfanilic acid and 1-amino-2-naphthol are generated. In this study, 0.32 mol of sulfanilic acid was generated from 1 mol of orange II degraded. Yet 50% of the

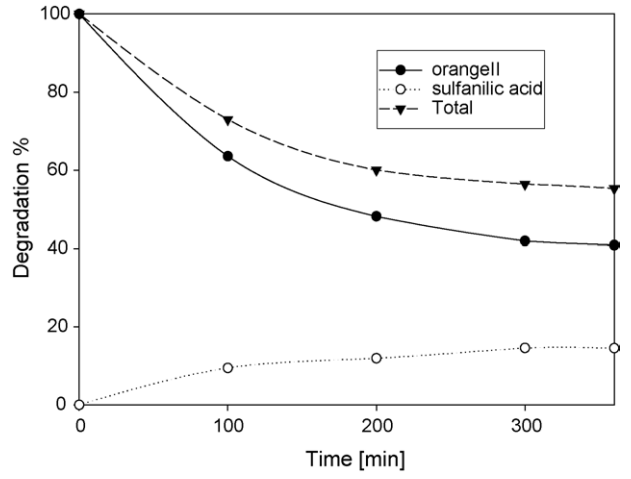

Fig. 7. The percentage of orange II not degraded and sulfanilic acid produced by the degradation of electroenzymatic method.

degradation products are not identified due to complex reaction, such as autoxidation Fig. 7.

4. Conclusions

The HRP-immobilized graphite felt showed a well-defined cyclic voltammograms, used to determine the optimum potential for the electro-generation of H_2O_2, which was found to be −0.5 V. The results of the experiments performed in this study proved that the use of an electroenzymatic treatment process is a viable approach for the degradation of azo dyes from aqueous solutions. In addition, when orange II was reduced using the electroenzymatic method, the azo bond was broken and the by-products consisted of an aromatic amine, sulfanilic acid, and other intermediate products.

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