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OXIDATIVE DEGRADATION AND DETOXIFICATION OF TEXTILE AZO DYE BY HORSERADISH PEROXIDASE ENZYME

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ABSTRACT

Horseradish peroxidase (HRP) can be readily extracted from horseradish roots. In this research, the application of acid blue-25 (AB-25) dye removal by low purity horseradish peroxidase extracted from horseradish root (crude HRP) was investigated. Parameters, such as amount of HRP, contact time, pH, H_2O_2 concentration, AB-25 concentration and temperature during enzyme-mediated dye degradation and detoxification, were studied. Results showed that decolorization of AB-25 at 75 min contact time, pH 7and 0.8 mM/L H_2O_2 for 1 U/ml crude and pure HRP were approximately 80 and 94%, respectively. Also, gradually adding of H_2O_2 and enzyme increased the turnover of the HRP compared to control. Polyethylene glycol (PEG) in various concentrations did not significantly affect the reduction of HRP inactivation. Toxicity test with *Daphnia magna* showed a reduction in toxicity after the enzymatic treatment. To meet the optimized conditions in the process, the ratios of dye/ enzyme and H_2O_2/dye, and sequences of the addition of reactants, should be considered.

KEYWORDS: Horseradish peroxidase; azo dye; enzymatic treatment; crude enzyme

ABBREVIATIONS

HRP: Horseradish peroxidase
AB-25: Acid Blue 25
PEG: Polyethylene glycol
ADMI: American Dye Manufactures Institute
U/ml: Unit enzyme/milliliter
4-AAP: 4-aminoantipyrine

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1 INTRODUCTION

Synthetic dyes have been extensively used in a broad range of industries. Over 7×10^5 tons and approximately 10,000 dyes and pigments are produced annually worldwide, about 10% of which may be found in wastewater [1]. Normally, colors are noticeable at a dye concentration of more than 1 mg/l; however, an average concentration of 300 mg/L has been reported in effluents from textile manufacturing processes [1-3]. Azo dyes are the major group of dyestuffs and have been extensively used in textile, printing, leather and cosmetics industries; they have been identified as the most problematic compounds in textile effluents due to their higher water solubility and lower degradability [2-4]. Therefore, the decolorization of wastewaters containing azo dyes prior to discharge is mandatory by environmental regulations in many countries.

Decolorization of industrial dyes can be achieved by physico-chemical methods, such as adsorption, precipitation or chemical degradation, and biological methods (aerobic and anaerobic); some of these methods are very expensive, which limits their application [2, 5-7]. The decolorization of the dyes through intermediates of different color has been proposed to be a series of multiple reactions. In case of anaerobic decolorization of azo dyes by some bacteria, arising intermediates and final products are carcinogenic and, mostly, more toxic than the starting dyes [2, 8, 9]. In recent years, enzymatic approach has attracted much interest in the removal of phenolic pollutants from aqueous solutions as an alternative strategy to the conventional chemical as well as microbial treatments that pose some serious limitations. Oxidoreductive enzymes such as peroxidases are participating in the degradation/removal of aromatic pollutants from various contaminated sites [10-12].

Peroxidases have been isolated from many species of plants, animals, and microorganisms. These enzymes are able to act on a variety of aromatic compounds in the presence of hydrogen peroxide [6, 13-15]. The enzymatic

treatment efficiency was found to be independent of the enzyme purity and, therefore, it was possible to utilize a crude enzyme preparation instead of a purified one. This feature leads to a significant reduction in treatment costs. The last research found that PEG greatly reduced enzyme requirements to achieve a removal of initial substrate [7, 16-20].

This work aims at developing an enzymatic process to remove toxic dye compounds from industrial wastewaters. There is no report about the application of crude HRP for the treatment of azo dyes. The present study focuses on the evaluation of parameters leading to degradation of AB-25 with concentrations in the range of 11-35 mg/L using crude enzyme prepared from horseradish roots. Reactions were conducted under several pH values (3-10), contact time, color, HRP (0.5-4 U/ml), H_2O_2 (0.2-1 µl/L mass concentration) and also the effect of PEG (5-100 mg/l) on batch system. According to the mechanism of enzymatic degradation of phenol adding mode can effect on turn over time of the enzyme [21], the effect of adding mode on the turn over time of the enzyme was studied. The experimental data of initial reaction rates were fitted to Michaelis-Menten equation. The acute toxicity tests with *Daphnia magna* were carried out to determine the detoxification capacity of the enzymatic treatment process.

2 MATERIALS AND METHODS

2.1 Materials

Low purity HRP was extracted from horseradish roots purchased from local vegetable market according to the procedure given by Bhunia *et al.* [22]. After cleaning with water, the roots were crushed in a wet grinder and the extract was centrifuged (6000g, 6 min, 4 ^oC). The resulting supernatant was dialyzed using 12KD membrane against 0.1M phosphate buffer (pH 7.4) at 4 ^oC. The dialyzed enzyme extract was stored at 4 ^oC and used in the treatment process; the activity of low purity before any treatment process was also assayed [16, 18, 23]. One step for purification of the low purity HRP by application of ammonium precipitation in 0-35% and 35-90% saturation of ammonium sulfate was carried out to increase purification of HRP.

AB-25 [1-amino-9,10-dihydro-9,10-dioxo-4-(phenylamino)-2-anthracenesulfonic acid, monosodiumsalt] (dye content 45%) was purchased from Aldrich and diluted in distilled water, up to a concentration of 100 mg/L, giving a stock solution from which aliquots were taken for the decolorization experiments. The dye concentrations were used without prior purification. A correlation between mg/L of dye and American Dye Manufactures Institute (ADMI) units was examined, and results show that there is a linear correlation between ADMI and concentration of AB-25: $ADMI = 47530X + 4.5$. In this equation, X is the concentration of AB-25 as g/L.

Aqueous solution of hydrogen peroxide (30% w/v, specific gravity 1.12), 4-aminoantipyrine, HRP and polyethylene glycol (average molecular mass of 1,500) were purchased from Sigma-Aldrich. All other chemicals used were of analytical grade.

2.2 Peroxidase activity assay

HRP enzyme activity was measured using phenol, 4-aminoantipyrine (AAP) and hydrogen peroxide as substrates. The approach was to provide all components, except enzyme near saturation concentration, so that the initial rate of reaction became directly proportional to the amount of enzyme present. The assay mixture contained 2.5 ml 9.6 mM of AAP, 1 ml 100 mM of phenol, 1 ml 2 mM of hydrogen peroxide, 4.5–5.0 ml 100 mM of phosphate buffer, pH7.4 and 0.5–1 ml enzyme solution. The rate of reaction was measured by monitoring the rate of formation of the products which absorbed light at a peak wavelength of 510 nm upon addition of the enzyme; thus, one unit of activity (U) used in this study is defined as the number of mM peroxide converted per min at pH 7.4 and 25 °C [17, 22, 24-26].

2.3 Experimental procedure

Mixed solution contains 0.021-1.69 ml of enzyme source (2.36 U/ml activity), 0.2-1 mM/L of H_2O_2, 10.4-31.5 mg/L of AB-25 that caused 500-1500 ADMI in solution, and pH 7.4. The enzymatic reaction was carried out under shaking in a horizontal shaker at 200 rpm about 5-70 min. The experiments were conducted at a constant temperature of 25 °C. Aliquots were removed every 5 min, being centrifuged at 6,000 rpm and submitted to analytical control.

2.4 Analytical process

The ADMI color value for each sample was measured via the following procedure: sample pH adjustment; centrifugation (6,000 rpm); absorbance measurement by spectrophotometry; and ADMI color calculation. For the pH adjustment step, two different pH values were chosen: original (environment) pH and pH 7.6 [27]. A Hach DR 5000 spectrophotometer was used to measure absorbances. Absorbances were measured at 31 wavelengths to calculate the ADMI index according to the APHA method.

2.5 Acute toxicity test with *Daphnia magna*

The acute toxicity tests using *Daphnia magna* were carried out according to the ABNT standards. The sensitivity tests were carried out with young organisms (6–24 h of life), which were not fed during the test period. For each concentration, 10 organisms were used in a 25-ml beaker, in duplicate for each concentration, along with the controls with dilution water (basic medium). The acute toxicity tests with the effluent samples had a duration of 24-96 h, and after this time of exposure, the number of immobile organisms was observed and noted. The organisms were considered to be immobile, if they did not show any mobility during 20 s of observation [28, 29]. In order to cal-

culate LC_{50}, probit analysis with SPSS (ver 11.5) was applied.

2.6 Modeling of the reactions

The initial reaction rates were then fitted using the Michaelis-Menten model with inhibition by the substrate [18]:

$$V_i = \frac{V_{max}.[H_2O_2]}{K_m + [H_2O_2] + K`.[H_2O_2]^2} \quad (1)$$

where, Vi and $[H_2O_2]$ are, respectively, the apparent rate of the dye consumption and the H_2O_2 initial concentration, while V_{max} is the apparent maximum reaction rate, K_m, the apparent Michaelis constant, and K`, the inhibition constant. The parameters of the kinetic model values $V_{max}$, $K_m$, and K` were estimated using the least-square approximation with MS Excel. In this procedure for estimating constants, all the parameters were optimized at the same time.

3 RESULTS AND DISCUSSION

3.1 Effect of contact time and HRP concentration

To determine the optimum contact time for AB-25 removal and HRP concentration, reaction mixture had 20 ml sample of 500 ADMI dye, 0.5-2 U/ml HRP and 1 mM H_2O_2 with phosphate buffer (pH 7.4) at room temperature (25 °C) being agitated for a period of 75 min. For every 5-min time interval, one sample was removed and analyzed for the residual dye concentration (Fig. 1). Results show that 70 min contact time and 1 U/ml of crude HRP enzyme led to 79% dye removal. After 70 min of contact time, a negligible dye removal was noticed. In this treatment conditions, 94% removal of AB-25 was achieved; that is more than crude HRP (about 75%) according to Fig. 1. The removal of an aromatic compound is dependent on the concentration of the enzyme added, considering that the catalyst and also the conversion depend on the contact time, and the respective optimum performance parameters (pH, temperature and substrate). The optimization of the quantity of enzyme was carried out aiming at a high efficiency of decolorization with a lower enzyme quantity used in the process. According to De Souza *et al.* [6], 5 min are sufficient to obtain a degradation of the dye Lanaset Blue 2R. However, Kim *et al.* [25] have reported that 45 min is the time required to catalyze the degradation of the dye Acid Black 10 BX, and that after 5 min, the removal reaction followed a slower process, and the decolorization was 67% with HRP in the free form. Sequencing experiments were performed for 70 min contact time and 1 U/ml crude HRP.

3.2 Effect of pH

In order to determine the effect of pH on AB-25 removal in 70 min contact time, it was varied from 5 to 9 in the mixture containing 1U/ml of crude HRP, and 1 mM H_2O_2 at 25 °C. The results of the experiments are shown in Fig. 2. These reactions reached the lowest remaining dye concentration at pH 7. So, the optimum pH was 7 for these experimental conditions. It was reported that the mechanism of HRP and H_2O_2 catalyzing organic pollutants, such as chlorophenols, is that the substrates can be converted into the free radical products by HRP and H_2O_2 attack, and the coupling reactions of the free radical products lead to polymerization [10]. The process of HRP catalyzing the dye removal may be similar to that process. So, we can say that the pH influenced the activity of enzyme and solubility of by-product that changes the action efficiency of crude HRP on substrates.

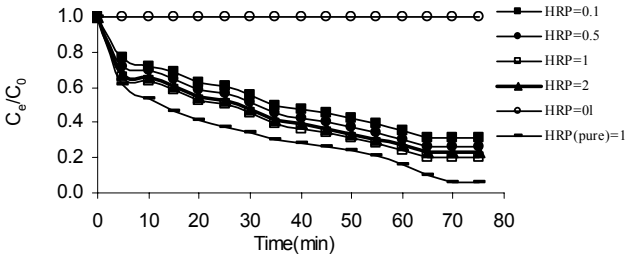

FIGURE 1 - Effect of the HRP initial concentrations on AB-25 removal in batch reactor (AB-25 = 10.4 mg/L; H_2O_2 = 0.8 mM; (0-2) U/ml pure HRP; pH 7, 25 ºC).

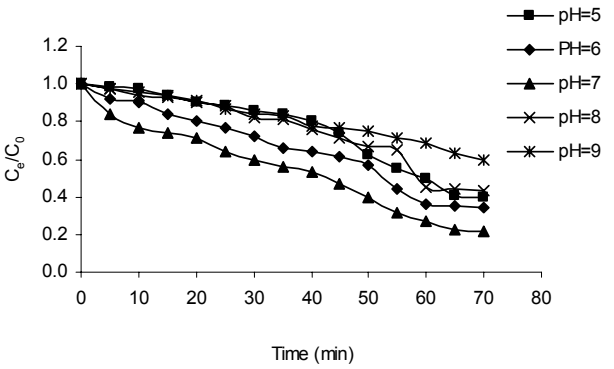

FIGURE 2 - Effect of pH on the efficiency of AB-25 removal.

3.3 Effect of H_2O_2 concentration

In order to determine the effect of H_2O_2 concentration on AB-25 removal, experiments under the same conditions as above were carried out (H_2O_2 0.2-1 mM). The results of the experiments are illustrated in Figs. 3 and 4. Results show that single HRP and H_2O_2 did not significantly decrease the remaining dye concentration from dye solutions. Two initial concentrations of 10.4, 31.5 mg/L of AB-25 were used to assess the effect of dye concentration on H_2O_2 consumption. A blank was made without H_2O_2 and after several min of reaction, the AB-25 concentration did not suffer any variation. Results show that optimum concentration of H_2O_2 was 0.8 mM and the concentration of substrate had no effect on the experiment.

3.4 Effect of temperature

The effect of temperature on dye removal was investigated at pH 7 over a temperature range of 10-75 °C (AB-25 500 ADMI, H_2O_2 0.8 mM, HRP 1 U/ml). The

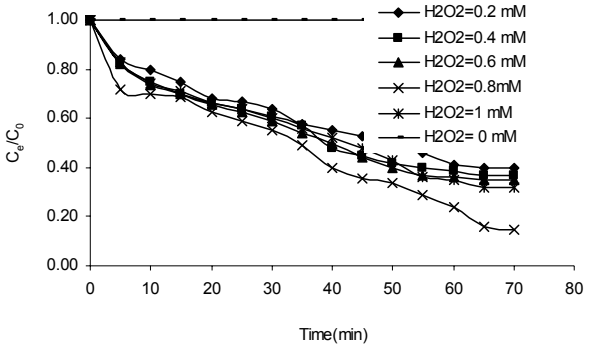

FIGURE 3 - Effect of the H_2O_2 initial concentration on the dye conversion in the reactor (AB-25 = 10.4 mg/L; HRP = 1 U/ml; pH 7 and 25 ºC).

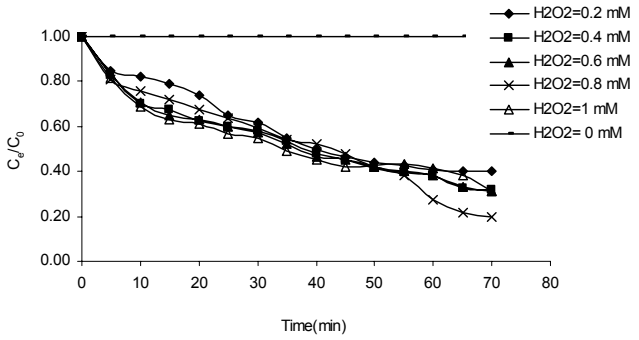

FIGURE 4 - Effect of the H_2O_2 initial concentration on the dye conversion in the reactor (AB-25 = 31.5 mg/L; HRP = 1 U/ml; pH 7, 25 ºC).

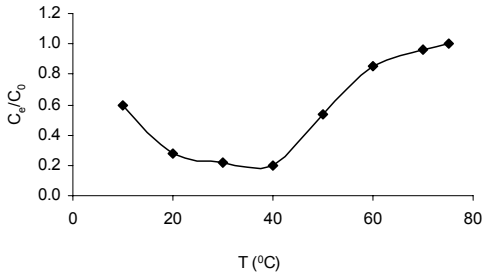

FIGURE 5 - Effect of temperature on AB-25 removal.

removal rates of enzymatic reaction depended on the temperature of reaction (Fig. 5). Results show that dye removal increased from 10 to 40 °C, and then decreased to 75 °C, which can be probably explained by the inactivation of HRP. So, the optimum temperature dye removal was 40 °C.

3.5 Effect of H_2O_2 and enzyme adding mode

To determine HRP and H_2O_2 adding mode, two samples (gradually adding H_2O_2 and enzyme and also whole amounts of H_2O_2 and enzyme) in enzymatic reaction process (AB-25 500 ADMI, H_2O_2 0.8 mM, HRP 1 U/ml, pH 7 and 70 min contact time at 25 °C) was investigated. Results show that gradually adding H_2O_2 and enzyme was better than adding the whole amounts of both. According to the mechanism of phenol polymerization, at high concentration of hydrogen peroxide, peroxidase effectively converts to a verdohemoprotein referred to as P-670 as it catalyzes oxidation of aromatic hydrogen donors (AH) in the catalytic cycle of the reaction. Suicide-peroxide inactivation has been shown to reduce the sensitivity and efficiency of peroxidase [21]. These results are shown in Fig. 6.

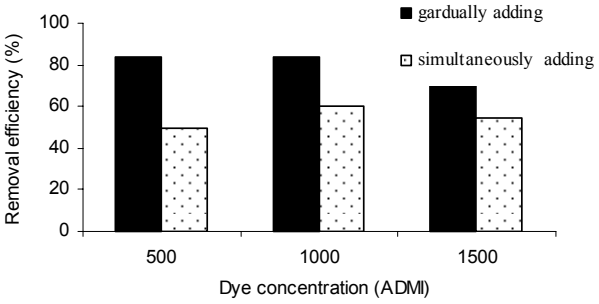

FIGURE 6 - Effect of H_2O_2 and enzyme adding mode.

3.6 Effect of PEG

HRP solution of 1 U/ml was prepared and its activity was measured using the peroxidase activity assay method. The activity was measured again, but in a buffer with PEG instead of buffer alone as specified in the assay [30]. Different PEG concentrations (up to 5 mg/L) in the cuvette were used to observe the dose effect. It was found that the average activity in the cuvette with PEG increased by about 9–11%, irrespective of PEG dose over a wide range of concentrations of PEG. These results are different from the other research, indicating that PEG caused increase in HRP enzyme usage. Another research reported that PEG greatly reduced enzyme requirements to achieve a removal of initial substrate such as phenol [30].

3.7 Effect of HRP removal of AB-25 on toxicity of solution

In the present study, toxicity was evaluated at concentrations of 10-30 mg/L for AB-25 to determine LC_{50} ranges. Also, control solutions (dye concentration of 0.0 mg/L) were conducted to confirm the accuracy of the test. The results indicate that the LC_{50} values of AB-25 for *Daphnia magna* are about 6.25 mg/L at 24 h, 4.97 mg/L at 48 h and 4.05 mg/L at 96-h tests. But after treatment of AB-25 by HRP, the toxicity was significantly decreased, and LC_{50} of AB-25 for *Daphnia magna* was about 13.35 mg/L at 24 h, 13.35 mg/L at 48 h and 11.62 mg/L at 96-h tests. This means, application of this process to detoxify AB-25, significantly decreased toxicity of solution. LC_{50} values of the solution before and after degradation with HRP are presented in Fig. 7.

3.8 Modeling of the reactions

The values of the fitted parameters for different dye concentrations are presented in Table 1. As it can be observed, the fittings are in good agreement with the experimental data. The results of the enzymatic elimination have the same trend as obtained by previous works [31].

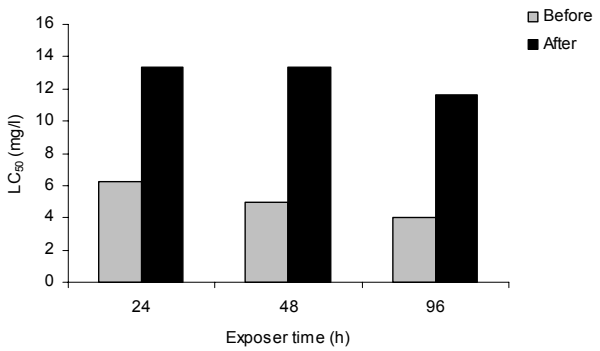

FIGURE 7 - Effect of HRP removal of AB-25 on toxicity of solution.

But the kinetic parameters cannot be compared with other literature values because the kinetic model used was different. The rate constants are different for each type of peroxidase, and they are functions of the pH, temperature, the kind of aromatic compound that is treated and, finally, of the kinetic model applied.

Although a good fitting of the experimental data of initial reaction rate was obtained using the kinetic model of Michaelis-Menten with inhibition, it was not possible to model globally.

TABLE 1 - Apparent Michaelis-Menten parameters for the reaction of HRP with AB-25 and H_2O_2 at optimum condition and different dye concentrations.

AB-25 (mg/L)	V_{max} (mM/min)	Km (mM)	K` (1/mM)	Cumulative error
10.4	1.17	0.43	0.35	0.002
21.05	2.57	0.63	0.62	0.011
31.5	3.37	1.37	0.8	0.016

4 CONCLUSIONS

The results of this study have demonstrated the applicability of using crude horseradish peroxidase (HRP) enzyme for treatment of an azo dye in aqueous solution. The main findings of this study are optimum conditions of the reaction: decolorization of the AB-25 at 75 min contact time, pH 7and 0.8 mM/L H_2O_2 for 1 U/ml crude and pure HRP was approximately 79 and 94%, respectively; also gradual adding of H_2O_2 and enzyme increased the turnover of the HRP compared to control (whole amounts H_2O_2 and enzyme at once). Excess peroxidase had no significant effect on the removal of the dye. The removal efficiency also increased until the optimum ratio was reached. PEG has no significant effect on reduction of HRP inactivation. There was reduction in toxicity after the enzymatic treatment of AB-25 in aqueous solutions. Crude HRP has a good efficiency for removal of AB-25 dye, and caused lower treatment costs relative to a pure source of HRP.

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