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Enzymatic Treatment and Detoxification of Acid Orange 7 from Textile Wastewater

Fathollah Gholami-Borujeni · Amir Hossein Mahvi · Simin Nasseri · Mohammad Ali Faramarzi · Ramin Nabizadeh · Mahmood Alimohammadi

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Abstract A crude preparation of horseradish roots was used as a low-purity source of horseradish peroxidase (HRP) in dye decolorization experiments. The technical feasibility of the process was studied in bench scale for enzymatic removal of acid orange 7 (AO7), a synthetic dye. Further studies were carried out to understand the effects of process parameters such as pH value, H_2O_2 level, concentrations of the synthetic dye, and HRP during enzyme-mediated dye degradation. Experimental data revealed that the concentration of AO7, pH of the aqueous phase, amount of the enzyme, and H_2O_2 level played significant roles on the overall enzymatic reaction. Polyethylene glycol, as an anti-inactivation of HRP, in various concentrations showed no significant effect on the decolorization. The experimental data of initial reaction rates were fitted using an analytical equation proposed by Michaelis–Menten. The acute toxicity tests using *Daphnia magna* exhibited that the enzymatic treatment significantly decreased the toxicity of the dye solution.

Keywords Acid orange 7 · Enzymatic treatment · Horseradish peroxidase · Horseradish root · Removal

Introduction

Textiles are made of a variety of materials and may contain a large number of chemicals that are employed during the production of fibers as preservative, finishing, and coloring agents, and these chemicals are sometimes released during normal wear or washing [1, 2]. Color

F. Gholami-Borujeni · A. H. Mahvi · S. Nasseri · R. Nabizadeh · M. Alimohammadi
School of Public Health and Center for Environmental Research, Tehran University of Medical Sciences, Tehran, Iran

A. H. Mahvi (✉)
National Institute of Health Research, Tehran University of Medical Sciences, Tehran, Iran
e-mail: ahmahvi@yahoo.com

M. A. Faramarzi
Department of Pharmaceutical Biotechnology, Faculty of Pharmacy and Biotechnology Research Center, Tehran University of Medical Sciences, P.O. Box 14155-6451, Tehran 14174, Iran

usually appears in low concentrations as the result of some specific chemical compounds, such as azo dyes, which represent the most common group of industrial dyes [3, 4]. More than 60–70% of more than 10,000 dyes used in the textile industry are azo dyes with azo linkage (R1–N=N–R2), where R1 and R2 are aromatic groups that, in some cases, can be substituted by sulphonated groups [5]. However, the products obtained from the cleavage of azo bonds are aromatic amines that are considered to be carcinogenic compounds [1]. The discharge of wastewater containing high concentration of aromatic dyes is a well-known problem associated with dyestuff activities. Their highly variable and complex chemical structures also make them difficult to remove by using conventional wastewater treatment systems [6–8]. The most commonly used color removal methods are physical (adsorption, filtration, and flotation), chemical (coagulation, oxidation, reduction, and electrolysis), and biological (aerobic and anaerobic). These methods are not entirely satisfactory in terms of cost, efficiency, and environmental impact [4, 9–11].

Recently, for the removal of phenolic pollutants from aqueous solutions, the enzymatic approach has attracted much interest as an alternative strategy to the conventional chemical as well as microbial treatments that impose some serious limitations [3, 12–15]. Oxidoreductive enzymes, such as peroxidases, are used in the degradation/removal of aromatic pollutants from various contaminated sites [9, 16–18]. Peroxidases have been isolated from many species of plants, animals, and microorganisms [4, 5, 13, 15]. These enzymes are able to act on a variety of aromatic compounds in the presence of hydrogen peroxide [3, 19–21]. The function of the latter is to oxidize the enzyme into a catalytically active form that is capable of reacting with the phenolic contaminant [17, 22, 23].

The polymeric products have limited water solubility and tend to precipitate readily [12]. The enzymatic treatment process has several limitations, including the prohibitive cost of treatment, the potential formation of residual products that remain in the aqueous phase, and enzyme turnover. Some benefits of enzymatic treatment are broad substrate specificity; effectiveness over a wide range of operating conditions including pH, temperature, salinity, and substrate concentrations; and the ability to remove other organic compounds by co-precipitation. Significant enzyme inactivation may also occur during the polymerization step. Free radicals generated in the catalytic cycle adsorb to the enzyme's active site and hinder the access of substrates. The semi-batch addition of H_2O_2 to maintain an optimized ratio between peroxide and horseradish peroxidase (HRP) concentrations was found to suppress this inhibition [21, 24]. By employing additives, such as polyethylene glycol (PEG), the apparent enzyme inactivation is alleviated to drastically reduce the amount of enzyme required. PEG combines with the polymers and is separated from the solution as precipitate [12, 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 [23].

The present study focused on the evaluation of parameters leading to the removal of AO7 in aqueous solutions by using a crude and low-purity source of horseradish peroxidase. The acute toxicity of the resulting dye solution was also examined using *Daphnia magna*.

Materials and Methods

Materials

Low-purity HRP was extracted from horseradish roots from a local vegetable market according to the procedure given by Bhunia and co-workers [9]. After washing the roots

with distilled water, they were crushed in a wet grinder without water and then the extract was centrifuged (6,000×*g*, 6 min, 4 °C). The resulting supernatant was dialyzed using 12 kDa membranes against a 0.1-M phosphate buffer (pH 7.4) at 4 °C. The dialyzed enzyme extract was stored at −20 °C and used later in the treatment process. The activity of the low-purity-enzyme was assayed prior to its use in removal experiments. One step of purification of the low-purity was carried out using precipitation in 0–35% and 35–90% saturation of ammonium sulfate [25].

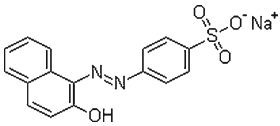
Acid orange 7 (85–95%) was purchased from Sigma and diluted in distilled water up to a concentration of 100 mg/l, giving a stock solution from which aliquots were taken for the decolorization experiments. The correlation between milligrams per liter of dye and American Dye Manufacturers Institute's (ADMI) color value was examined and the results show that there is linear correlation between ADMI and the concentration of AO7 according to the following equation $\mathrm{ADMI} = 42,200X + 22$, in which X is grams per liter of AO7. Some of the chemical properties of AO7 are presented in Table 1 [1].

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

Peroxidase Activity Assay

HRP enzyme activity was measured using phenol, 4-aminoantipyrine (AAP), and hydrogen peroxide as substrates. The approach in this assay was to provide all components, except the enzyme, at near saturation concentration so that the initial rate of reaction was directly proportional to the amount of enzyme present. The assay mixture contained 250 μl and 9.6 mM AAP, 100 μl and 100 mM phenol, 100 μl and 2 mM hydrogen peroxide, 450–500 μl and 100 mM phosphate buffer, pH 7.4, and 50–100 μl enzyme solution. The rate of reaction was measured by monitoring the rate of formation of the products that absorbed light at a peak wavelength of 510 nm upon the addition of the enzyme; thus, one unit of activity (U) is defined as the number of mmol peroxide converted per min at pH 7.4 and 25 °C [6, 9].

Table 1 Chemical properties of AO7

Properties	Value
Name	Acid Orange 7 (AO7)
Molecular Structure	
Molecular Formula	$C_{16}H_{11}N_2NaO_4S$
Molecular Weight	350.32
Water solubility	116 g/L (30 ºC)

Experimental Procedure

The experiments were carried out at 0.021–1.69 ml of enzymatic solution with activity 2.36 U/ml, 0.2–1 mmol/l of H_2O_2, and 11.37–35.02 mg/l of AO7, respectively, which caused 500–1,500 ADMI color value in the solution in the presence of a phosphate buffer (pH 7.4). The enzymatic reaction was carried out in a horizontal shaker at 200 rpm for 5–70 min. The experiments were conducted at a constant temperature of 25 °C. Aliquots were removed every 5 min, centrifuged at 6,000 rpm, and then submitted to analytical control.

Analytical Process

The ADMI color value for each sample was measured by the following procedure: sample pH adjustment, centrifuge (6,000 rpm), spectrophotometer, and ADMI measurement. For the pH adjustment step, two different pH values were chosen: original (ambient) pH and pH 7.6 [26]. For the spectrophotometer step, a Hach DR 5000 was used. For the ADMI measurement step, 31 WL ADMI color values were measured according to the APHA method.

Acute Toxicity Test with *D. magna*

The acute toxicity tests with *D. magna* were carried out according to the ABNT norms. 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 the dilution water (basic medium). The acute toxicity tests with the effluent samples had durations of 24–96 h; and after this time of exposure, the number of immobile organisms was observed and noted. The organisms were considered immobile if they did not show any mobility during 20 s of observation [11, 27, 28]. For the calculation of LC_{50}, probit analysis, SPSS (ver. 11.5) was applied.

Results and Discussion

The peroxidase activity measured for horseradish roots was approximately 2.36 U/ml. Mohan et al. [5] reported that HRP extracted from horseradish roots was found to contain 2.94 U/ml of the enzyme after dialysis. Results show that ammonium precipitation caused increased activity of peroxidase to 18.4 U/ml by 55% saturation of ammonium sulfate. These results indicated that horseradish root distillate that has low activity could be concentrated by the ammonium precipitation method.

Effect of HRP Concentration

The effect of the initial HRP concentration on the removal of AO7 was analyzed. For this subject, a 100-ml solution containing HRP of 0.05–2 U/ml (step feed), 11.32 mg/l of AO7, and H_2O_2 of 1 mM (step feed) in a phosphate buffer at pH 7.4 at a room temperature of 25 °C was shaken at 200 rpm for 5–70 min. The samples were taken every 5 min from the solution, centrifuged at 6,000×*g*, and submitted to analytical control by spectrophotometer according to the ADMI method. As shown in the Fig. 1, the removal of AO7 increased the

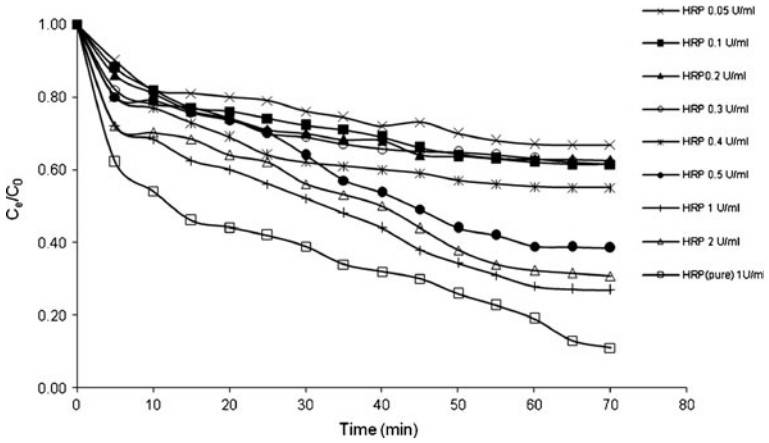


Fig. 1 Influence of the HRP initial concentration (0.05–2 U/ml) on the AO7 conversion in a batch reactor. AO7—11.32 mg/l, [H_2O_2]—1 mM, pH 7.4, and temperature of 25 °C

HRP concentration increased. In this case, for an initial concentration of AO7 of 11.32 mg/l and an H_2O_2 initial concentration of 1 mM, only 33% of AO7 conversion was obtained for a HRP initial concentration of 0.05 U/ml, whereas the AO7 removal increased to approximately 73% with a concentration of 1 U/ml. The low conversion of AO7 at low concentrations of HRP can be attributed to the inactivation of the enzyme, which may be the result of the interaction of the phenoxy radicals with the enzyme's active center and because of the inhibition by H_2O_2 [29]. This increase in the conversion of AO7 with an augmentation of HRP concentration indicated that the conversion of AO7 to other compounds is through the formation of free radicals. In a HRP concentration of 2 U/ml, the increase of HRP concentration does not increase the decolorization at the end of contact time, and this may be caused by enzyme inactivation. Mohan et al. [5] reported that the enzyme dose was found to have a significant influence on the dye removal reaction. The increase in the HRP dose from 0.735 U/ml to 2.205 U/ml might have resulted in a gradual increase in the Acid Black 10 BX removal rates (62–84%). Pure HRP has 89% color removal efficiency at 1 U/ml, which is higher than that for low-purity HRP.

Influence of the Hydrogen Peroxide Initial Concentration

The experiments to study the influence of the H_2O_2 initial concentration were carried out in batch conditions. The concentration of HRP was kept in 1 U/ml in all cases. The removal of the AO7 was studied for concentrations of H_2O_2 varying from 0.2 to 1 mM (step feed), while three initial concentrations of AO7 initial concentrations were used (11.32, 23.17, and 35.02 mg/l). A blank was made without H_2O_2 and, after several hours of reaction, the AO7 concentration did not suffer any variation. In all experiments, the solution turned dark brown immediately after the addition of the H_2O_2. More dark solutions were obtained with higher initial concentrations of H_2O_2, but when an excess of H_2O_2 was added, a lighter-colored solution was obtained. This fact is due to the amount of products formed increasing with the amount of H_2O_2 and, therefore, the inactivation of the enzyme provokes lower levels of conversion and a lower coloration of the solution. These results were also reported by Nicell and Wright [29]. The results of the AO7 at three initial concentrations of 11.32, 23.17, and 35.02 mg/l removal are illustrated in Figs. 2, 3, and 4.

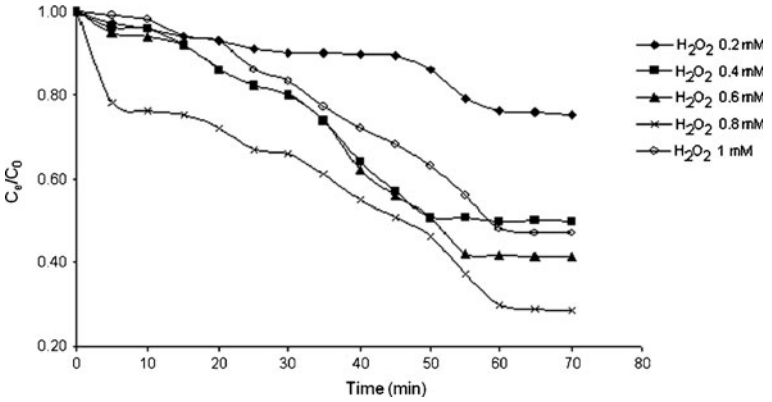


Fig. 2 Influence of the H_2O_2 initial concentration on the dye conversion in the reactor. [AO7]—11.32 mg/l, HRP—1U/ml, pH 7, and temperature of 25 °C

The results indicate that an H_2O_2 concentration of 0.8 mM is the optimum concentration for 1U/ml of this HRP, and there is no relation between the concentration of substrate and H_2O_2.

Effect of pH

The effect of pH on the efficiency of AO7 removal is shown in Fig. 5. Enzymes have an optimum pH range at which their activity is maximized. pH optimization studies were carried out on the AO7 dye by varying the aqueous phase pH of the reaction mixture between a pH from 5 to 9 by keeping the dye concentration (11 mg/l), enzyme concentration (1 U/ml), H_2O_2 dose (0.8 mM), and contact time (75 min) constant. It is observed that about 73% of the dye was removed due to a HRP catalyzed reaction at an aqueous phase of pH 7 with the specified experimental conditions. According to Lui et al. [9], 5 min and pH 5 are optimum for obtaining the degradation of the dyes bromophenol blue and methyl orange, with decolorizations of 100% and 80%, respectively. In a pH above and below 7, dye removal dropped significantly.

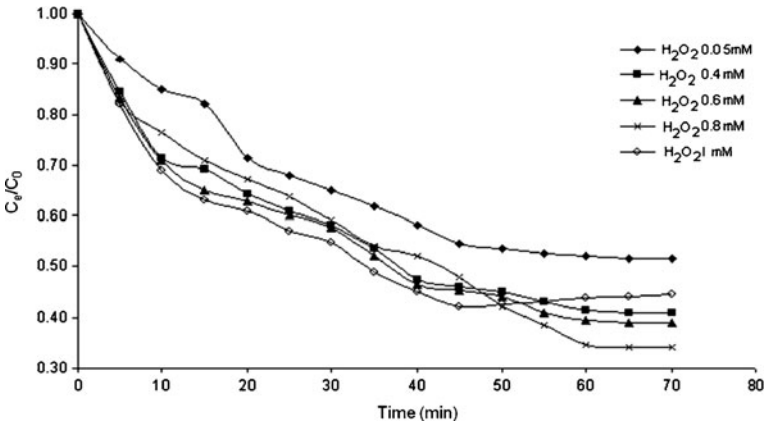


Fig. 3 Influence of the H_2O_2 initial concentration on the dye conversion in the reactor. [AO7]—23.17 mg/l, HRP—1U/ml, pH 7, and temperature of 25 °C

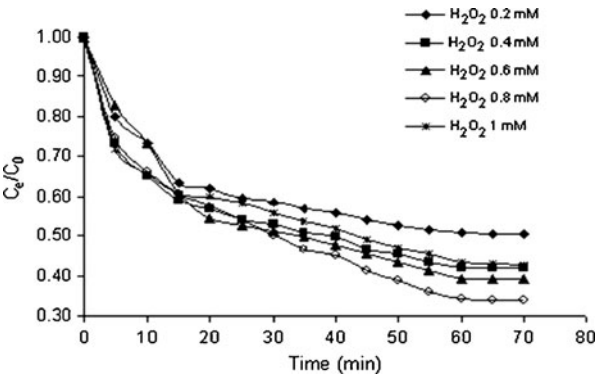


Fig. 4 Influence of the H_2O_2 initial concentration on the dye conversion in the reactor. [AO7]—35.02 mg/l, HRP—1 U/ml, pH 7, and temperature of 25 °C

Kinetic Study

Order of Reaction

The determination of the reaction order was carried out by curve fitting. A plot of Ln C/C_0 versus time showed that these enzymatic reactions occur in the first-order reaction by R^2=0.93. Determining reaction order by plotting is shown in Fig. 6.

The experimental data of the initial reaction rates were fitted using an analytical equation proposed by Michaelis–Menten that is usually used in enzymatic treatments. The Eq. 1 is defined by Cornish-Bowden [22]:

$$V_i = \frac{V_{max}[H_2O_2]}{K_m[H_2O_2]} \quad (1)$$

Where the variables V_i and $[H_2O_2]$ are the apparent rate of color removal and the H_2O_2 initial concentration, respectively. The apparent maximum reaction rate, V_{max}, and the apparent Michaelis constant K_m were estimated by the least-squares approximation with the solver of MS Excel according to Fig. 7. The Table 2 presents the values obtained for the kinetic parameters.

Michaelis–Menten Model with Inhibition by Substrate

The initial reaction rates were then fitted using another kinetic model, the Michaelis–Menten model, with inhibition by the substrate. The kinetic data as a function of the H_2O_2

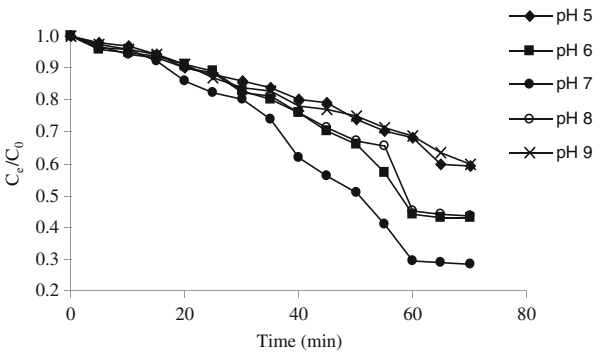


Fig. 5 Effect of pH on the efficiency of AO7 removal

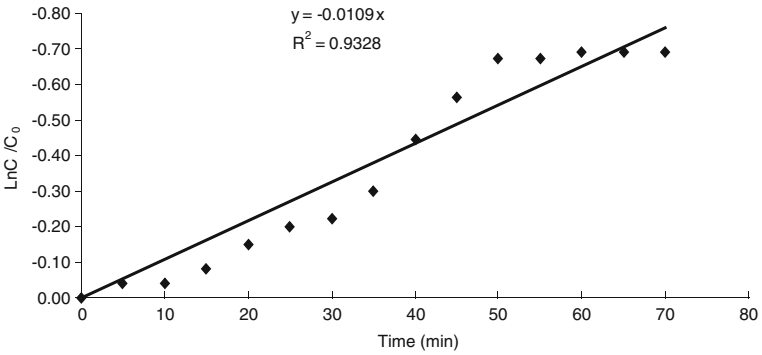


Fig. 6 Determining reaction order by plotting (first-order reaction)

concentration and the initial concentration of AO7 were studied. The initial rate (V_i) was calculated according to Eq. 2. H_2O_2 concentration and was fitted to the Michaelis–Menten equation with inhibition by the substrate [22]. The Michaelis–Menten equation with inhibition by the substrate is presented at Eq. 2:

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

Where the variables V_i and $[H_2O_2]$ are, respectively, the apparent rate of the dye removal 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 V_{max}, apparent maximum reaction rate, K_m, apparent Michaelis constant, and K', inhibition constant were estimated by the least-squares approximation with the solver of MS Excel. In this procedure for estimating constants, all the parameters were optimized at the same time. The calculated values of the fitted parameters for different dye concentrations are presented in Table 3.

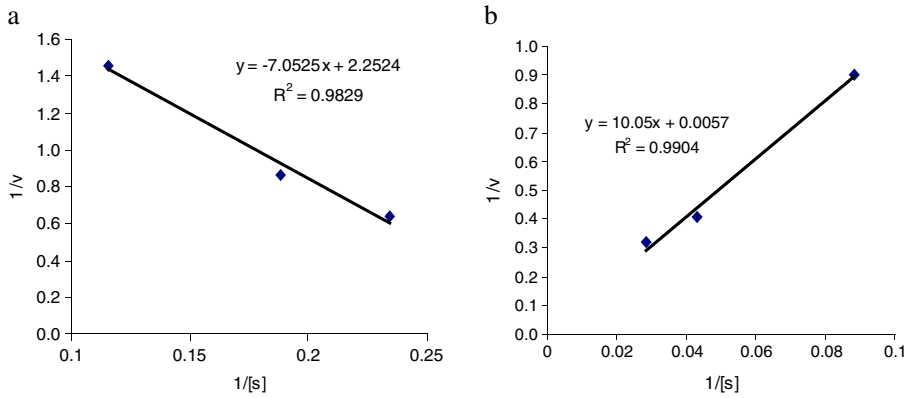


Fig. 7 **a** Michaelis–Menten model and **b** Michaelis–Menten model with inhibition by substrate

Table 2 Apparent Michaelis–Menten parameters for the reaction of HRP with color and H_2O_2

AO7 (mg/l)	V_{max} (mM/min)	K_m (mM)	Cumulative error
11.32	0.69	0.08	0.014
23.17	1.17	0.22	0.034
35.02	1.58	0.37	0.083

Effect of PEG

A HRP solution of 1 U/ml was prepared and its activity was measured using the assay. The activity was measured again, but in a buffer with PEG instead of a buffer alone as specified in the assay. 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 the PEG dose over a wide range of concentrations of PEG. These results are shown in Fig. 8.

Toxicity Assay

In the present study, toxicity was evaluated at concentrations of 0.8–10.0 mg/l for AO7 to determine LC_{50} ranges. Also, control solutions (concentration of 0.0 mg/l) were conducted to confirm the accuracy of the test. The results indicate that the LC_{50} of AO7 for *D. magna* is about 6.50 mg/l at 24 h, 4.65 mg/l at 48 h, and 3.72 mg/l at 96 h tests. But after the treatment of AO7 with HRP, this process can significantly decrease the toxicity of AO7 LC_{50} of AO7 for *D. magna* is about 11.58 mg/l at 24 h, 9.17 mg/l at 48 h, and 26.85 mg/l at 96 h. This means that the application of this process for the detoxification of AO7 significantly decreases the toxicity of the solution. LC_{50} of the AO7 data before and after degradation with HRP is presented in Fig. 9.

Conclusion

A horseradish root distillate that has low activity could be concentrated by the ammonium precipitation method and then used for the treatment process. Peroxidase enzymes from low-purity sources such as horseradish roots crude preparation showed good potential for azo dye degradation. The enzymatic removal of AO7 performed in the batch reactor obtained high degrees of conversion for the three initial concentrations of colors tested. Pure HRP has 89% color removal efficiency at 1U/ml, which is more than low-purity HRP (73%) that may be affected by other compounds present in distillate horseradish root. The

Table 3 Apparent Michaelis–Menten with inhibition parameters for the reaction of HRP with AO7 and H_2O_2

AO7 (mg/l)	V_{max} (mM/min)	K_m (mM)	K' (1/mM)	Cumulative error
11.32	1.15	0.42	0.34	0.001
23.17	2.54	0.68	0.61	0.01
35.02	3.27	1.45	0.71	0.017

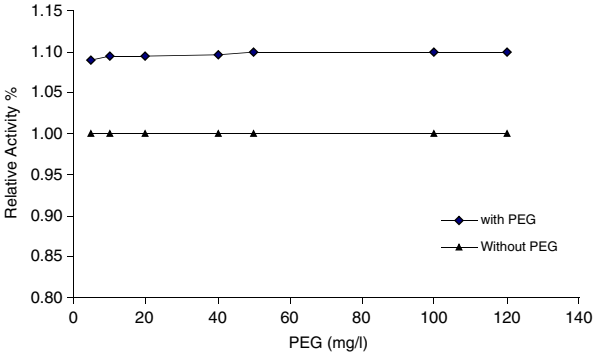


Fig. 8 Effect of PEG on activity of HRP

optimum H_2O_2 initial concentration to achieve the highest conversion of AO7 must be 0.8 mM. The excess of hydrogen peroxide in the mixture inhibits the catalytic activity of the enzyme through the conversion of peroxidase to inactive forms, thus provoking a reduction of the AO7 conversion. Optimum pH for the removal of dye is 7. Enzymatic reactions occur in the first-order reaction by R^2=0.93. The enzymatic reaction in the batch reactor follows the Michaelis–Menten equation with inhibition when the kinetic data is obtained as a function of the initial H_2O_2 concentration. But different kinetic parameters were found for different initial AO7 concentrations.

An alternative kinetic model based on Michaelis–Menten with inhibition was proposed. The equation of the apparent reaction rate for the 35.02 g/l of dye is Eq. 3:

$$V_i = \frac{3.27.[H_2O_2]}{1.45 + [H_2O_2] + 0.71.[H_2O_2]^2} \tag{3}$$

It was found that the average activity in the reactor with PEG increased by about 9–11% irrespective of the PEG dose over a wide range of concentrations (5–100 mg/l) of PEG. The acute toxicity tests with *D. magna* showed that the enzymatic treatment of AO7 by the application of low-purity horseradish peroxidase significantly decreases the toxicity of the solution.

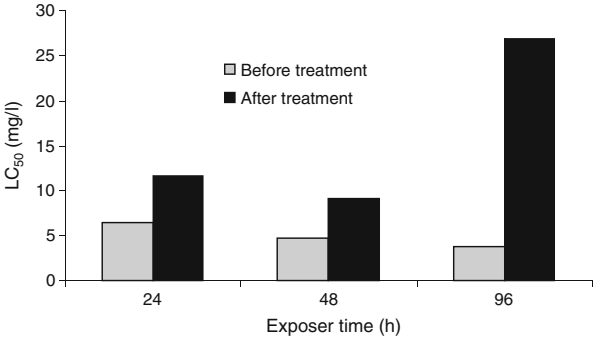


Fig. 9 Acute toxicity of AO7 to *Daphnia magna* before and after treatment

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