Enzymatic Degradation of Congo Red by Turnip (*Brassica rapa*) Peroxidase

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The enzyme peroxidase is known for its capacity to remove phenolic compounds and aromatic amines from aqueous solutions and also to decolourize textile effluents. This study aims at evaluating the potential of a turnip (*Brassica rapa*) peroxidase (TP) preparation in the discolouration of textile azo dyes and effluents. An azo dye, Congo Red (CR), was used as a model pollutant for treatment by the enzyme. The effects of various operating conditions like pH value, temperature, initial dye and hydrogen peroxide concentrations, contact time, and enzyme concentration were evaluated. The optimal conditions for maximal colour removal were at pH 2.0, 40 °C, 50 mM hydrogen peroxide, 50 mg/l CR dye, and TP activity of 0.45 U/ml within 10 min of incubation time. Analysis of the by-products from the enzymatic treatment by UV-Vis and IR spectroscopy showed no residual compounds in the aqueous phase and a precipitate of polymeric nature.

Key words: Congo Red, Discolouration, Peroxidase

Introduction

Waste water effluents from various industries including dyeing, textile, dye manufacturing, leather, cosmetics, food processing, and paper are considered main sources of dye pollution (Bhatnagar and Jian, 2005). There are more than 10^5 kinds of commercially available dyes with over $8 \cdot 10^5$ metric tons of dyestuff produced annually of which at least 10% are lost and released into industrial effluents (Palmieri *et al.*, 2005). Nearly half of all known dyes are azo dyes which makes them the most abundant group of synthetic dyes (Selvam *et al.*, 2003). These dyes are hardly degraded in the environment due to their resistance to oxidizing agents, light, and water (Meyer, 1981; O'Neill *et al.*, 1999). Textile waste waters are characterized as having a strong colour, since some of the initial dye (10–15%) is not fixed to the fiber during the dyeing process, thus being released into the effluents. When released into the environment without any treatment, they can cause serious contamination problems, decreasing water transparency and, consequently, inhibiting the penetration of solar radiation and decreasing photosynthesis.

Treatment of dye-based effluents is considered a challenge to environmentalists. The main techniques described in the literature for the discolouration of wastewaters involve adsorption, precipitation, chemical degradation, electrochemical, photochemical, and biodegradation processes, among others (Guaratini and Zanoni, 2000). Microbial discolouration has been proposed as a cheaper and less environmentally aggressive alternative (Mohorcic *et al.*, 2006). However, these procedures have not been widely used because of high cost, formation of hazardous by-products, and high energy requirement (Hai *et al.*, 2007).

Recently, enzymatic approaches have attracted much interest in the removal of phenolic pollutants from aqueous solutions as a strategy alternative to the conventional chemical as well as microbial treatments that pose some serious limitations (Husain and Jan, 2000; Duran and

Esposito, 2000; Yousefi and Kariminia, 2010). Oxidoreductive enzymes such as peroxidases and polyphenol oxidases participate in the degradation/removal of aromatic pollutants from various contaminated sites. These enzymes can act on a wide range of substrates and can also catalyze the degradation/removal of organic pollutants present at very low concentration at the contaminated site (Matto and Husain, 2009a). Considering the potential of these enzymes in treating phenolic compounds (Quintanilla-Guerrero *et al.*, 2008), several microbial and plant peroxidases and polyphenol oxidases have been considered for the treatment of dyes but none of them has been exploited on a large scale due to low enzymatic activity in biological materials and the high cost of purification (Bhunia *et al.*, 2001; Shaffiqu *et al.*, 2002). Enzymes can act on specific recalcitrant pollutants to be removed by precipitation or transformation into other innocuous products (Verma and Madamwar, 2002; Regalado *et al.*, 2004).

In this study, a peroxidase preparation from turnip (*Brassica rapa*) (TP) was used in the transformation of Congo Red (CR) (Fig. 1) dye, which is commonly found in the contaminated aquifers of textile industries (Zhu *et al.*, 2009; Chatterjee *et al.*, 2007). This study documents TP- and H_2O_2-mediated oxidation of CR dye and establishes some parameters of the reaction.

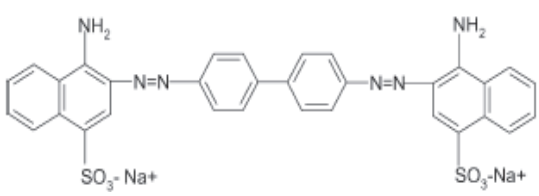

Fig. 1. Chemical structure of Congo Red.

Material and Methods

Dye

Congo Red dye (C.I., 22120; MW, 696.67 g/mol; λ_{max}, 499 nm; dye content, 97%) was supplied by Sigma-Aldrich (St. Louis, MO, USA). It belongs to the class of di-azo compounds, and its structure is shown in Fig. 1 (Purkait *et al.*, 2007). The aqueous solution of the dye was prepared just prior to the experiments by dissolving the requisite amount of dye in distilled water.

Extraction of TP

Peroxidase was extracted from turnip (*Brassica rapa* L.) obtained locally (Matto and Husain, 2009b; Kulshrestha and Husain, 2007). Turnip (100 g) was cleaned and crushed in a wet grinder with the addition of 200 ml distilled water. The homogenate was filtered through four layers of cheesecloth. The filtrate was subjected to fractionation by adding acetone. The mixture was stirred overnight at 4 °C to obtain maximum precipitation. The precipitate was collected by centrifugation at 4000 x *g* in a Remi R-24 cooled centrifuge (Remi Laboratory Instruments, Mumbai, India). The resulting pellet was redissolved in 100 mM sodium phosphate buffer, pH 6.0.

Enzyme assay

TP activity was assessed by the 4-aminoantipyrene method using phenol and H_2O_2 as substrates and 4-aminoantipyrene as chromogen (Bhunia *et al.*, 2001). The assay was performed at 25 °C in phosphate buffer (pH 6.0) containing 2 mM phenol, 0.2 mM 4-aminoantipyrene and 2 mM H_2O_2 in a total volume of 5.2 ml (1 ml of dye + 1 ml of H_2O_2 + 3 ml of buffer + 0.2 ml of enzyme). The rate of H_2O_2 consumption in the assay was calculated from the rate of formation of the coloured product at λ_{max} = 517 nm and a molar absorptivity of 5680 l/(mol cm).

One unit of activity (U) is defined as μmol H_2O_2 consumed per min.

Screening of peroxidase activity was similarly done on other plant sources (zucchini, cabbage, sweet potato, red radish, cauliflower, soybeans, and potato). Peroxidase was assayed in crude extracts in order to select the source giving the highest activity.

Quantitative determination of dye concentration

The dye concentration was determined spectrophotometrically at λ_{max} = 500 nm. After TP treatment, the sample was centrifuged and the residual dye concentration was determined in the supernatant (Kulshrestha and Husain, 2007).

Enzymatic Congo Red degradation

Initially, kinetics were determined in a series of vials containing 40 mg/l dye, 0.6 U TP, and 20 mM H_2O_2 and by varying the pH value of the aqueous phase of the reaction mixture between

2 and 10. After incubation for 1 h, followed by centrifugation at 4000 x *g* for 5 min at 24 °C, the residual dye concentration in the supernatant was determined. Subsequently, incubation time, dye concentration (5–40 mg/l), H_2O_2 concentration (0.5–200 mM), and TP concentration (0.03–3 U/ml) were varied to determine the optimal conditions for dye removal. All experiments were done in triplicate. Control (blank) experiments were performed without TP to assess abiotic degradation via photocatalytic or chemical processes.

Calculation of the yield of discolouration

The percentage of discolouration after enzymatic treatment was defined as:

$$\textit{dye removal } (R) = \frac{(A_0 - A_e)}{A_0} \cdot 100\%,$$

where A_0 and A_e are the absorbances before and after enzymatic treatment, respectively (Matto and Husain, 2009a, b).

Analysis of degradation products

After enzymatic CR discolouration, the reaction mixture was centrifuged, and the absorbance spectrum of the supernatant was determined in the UV-Vis region. The solid precipitate was vacuum-dried and analysed by infrared spectroscopy (FTIR) in the range of 4000–400 cm^{-1}. The spectra were compared to those of CR.

Results and Discussion

Extraction of peroxidase

Extraction of peroxidase was performed on different plant sources, taking into account their availability and cost at local markets, to identify the source of the highest peroxidase activity. As seen in Table I, the highest peroxidase activity was found in *Brassica rapa* which was therefore chosen as the source of peroxidase for dye degradation.

Table I. Peroxidase activity in different plant materials.

Peroxidase source	Enzymatic activity [U/ml]
White turnip (*Brassica rapa*)	3.406
Zucchini (*Cucurbita pepo*)	2.189
Cabbage (*Brassica oleracea* var. *capitata*)	0.865
Sweet potato (*Ipomea batatas*)	0.481
Red radish (*Raphanus sativus*)	0.181
Cauliflower (*Brassica oleracea* var. *botrytis*)	0.074
Potato (*Solanum tuberosum*)	0.026

pH

The dye was most effectively discoloured at pH 2.0 (95% during 1 h), and there was a significant drop of activity above pH 5.0 (Fig. 2), similar to findings for horseradish peroxidase (Buhnia *et al.*, 2001).

Incubation time

In mixtures containing 5 ml of dye solution (40 mg/l), 0.6 U/ml of enzyme, 20 mM H_2O_2 at pH 2.0, and a temperature of 24 °C, the reaction was rapid during the first 10 min, and then leveled off, until there was no further reaction after about 1 h of incubation (Fig. 3).

Temperature

The reaction rate was little affected by temperatures between 20 and 40 °C, but decreased sharply at higher temperatures (Fig. 4). A temperature of 25 °C was therefore chosen.

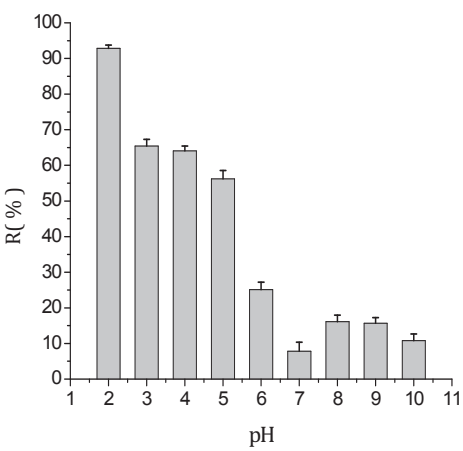

Fig. 2. Effect of pH value on the discolouration of Congo Red (40 mg/l) in the presence of 0.6 U/ml enzyme and 20 mM H_2O_2, at 24 °C for 1 h. *R*, yield.

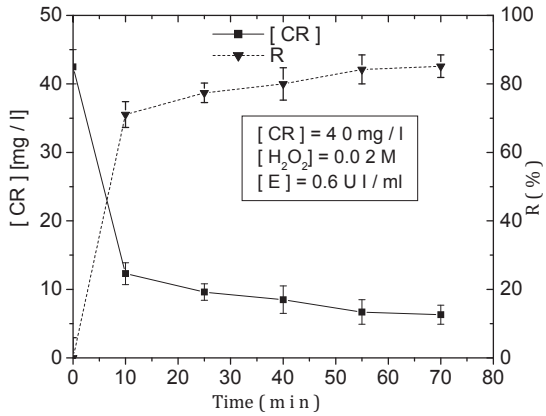

Fig. 3. Discolouration of Congo Red as a function of time. Other conditions as in Fig. 2.

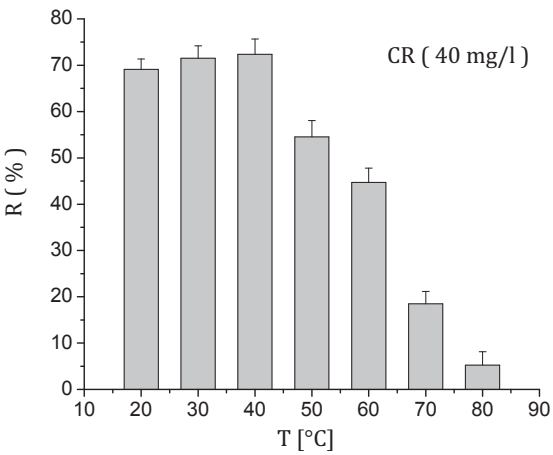

Fig. 4. Discolouration of Congo Red as a function of temperature. Other conditions as in Fig. 2.

Dye concentration

When dye concentrations were varied between 10 and 100 mg/l, keeping the other parameters at constant values, the initial velocity V_0 was proportional to the concentration of the substrate until reaching a maximum at a dye concentration of 50 mg/l (Fig. 5). Further increase in dye concentration was accompanied by a decrease in the rate V_0, obviously due to substrate inhibition. On the other hand, the percent yield R did not significantly change with increasing substrate concentration until 50 mg/l dye, with a rapid decrease at higher concentrations (Fig. 5). The optimal dye concentration was therefore 50 mg/l, giving the highest yield at the highest initial rate.

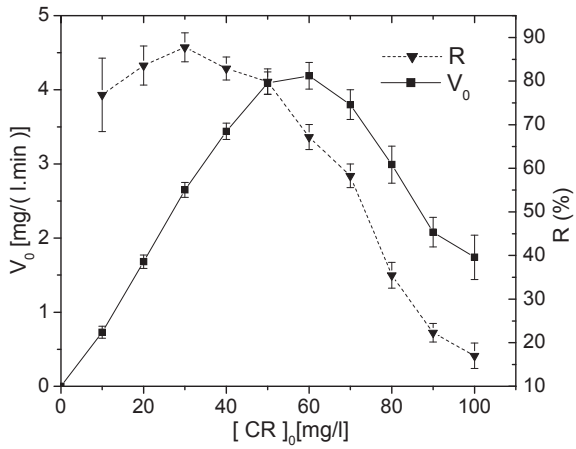

Fig. 5. Effect of substrate (Congo Red) concentration on the initial velocity V_0 and yield R of Congo Red discolouration. Other conditions as in Fig. 2.

H_2O_2 concentration

Varying the H_2O_2 concentration from 0.5 to 200 mM in the reaction mixture, keeping all other experimental conditions at their optimal values, gave the highest initial rate and yield at 50 mM H_2O_2 (Fig. 6), in agreement with the finding of other authors (Bhunia *et al.*, 2001; Kulshrestha

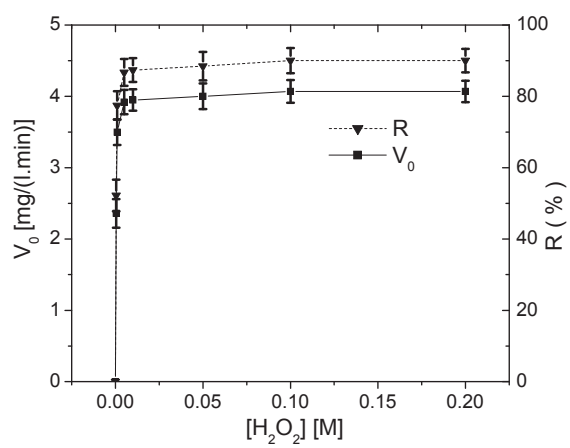

Fig. 6. Effect of substrate (H_2O_2) concentration on Congo Red (50 mg/l) discolouration during a 10-min incubation time. Other conditions as in Fig. 5.

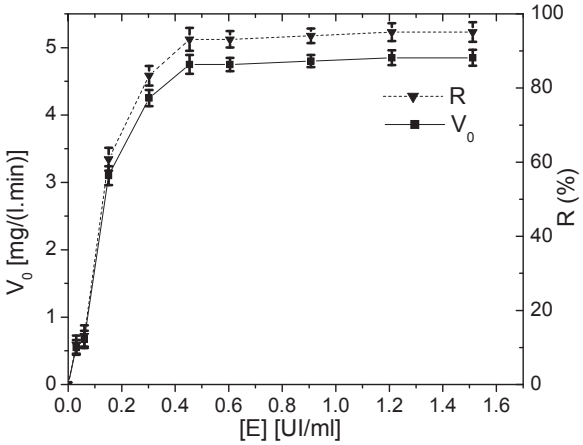

Fig. 7. Effect of enzyme concentration on Congo Red (50 mg/l) discolouration; 50 mM H_2O_2, pH 10.0, 10 min.

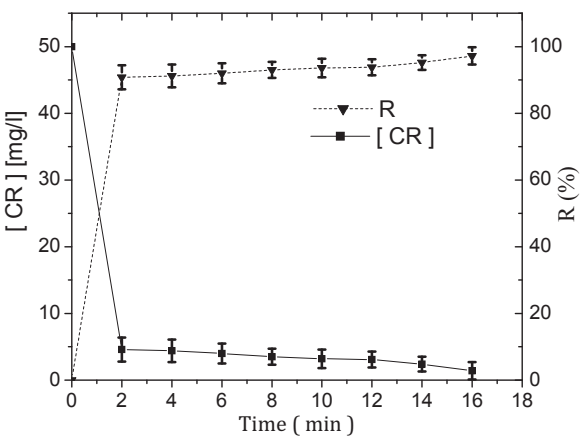

Fig. 8. Degradation of Congo Red under optimized conditions (50 mg/l Congo Red, 0.45 U/ml TP, 50 mM H_2O_2, pH 2.0, and 25 °C).

and Husain, 2007; Matto and Husain, 2009a, b; Ulson de Souza *et al.*, 2007; Maddhinni *et al.*, 2006). This concentration was therefore chosen for standard conditions.

Enzyme concentration

Initial velocity and percent yield of the reaction increased with enzyme concentration up to about 0.45 UI/ml of enzyme and did not change further at higher concentrations (Fig. 7).

Time course of CR discolouration under optimized conditions

Under optimized conditions, 90% of the dye were degraded within the first 2 min, and total discolouration of the reaction mixture was achieved after about 16 min (Fig. 8).

Product analysis

The absorption spectra of reaction mixtures before and after 96% discolouration are shown

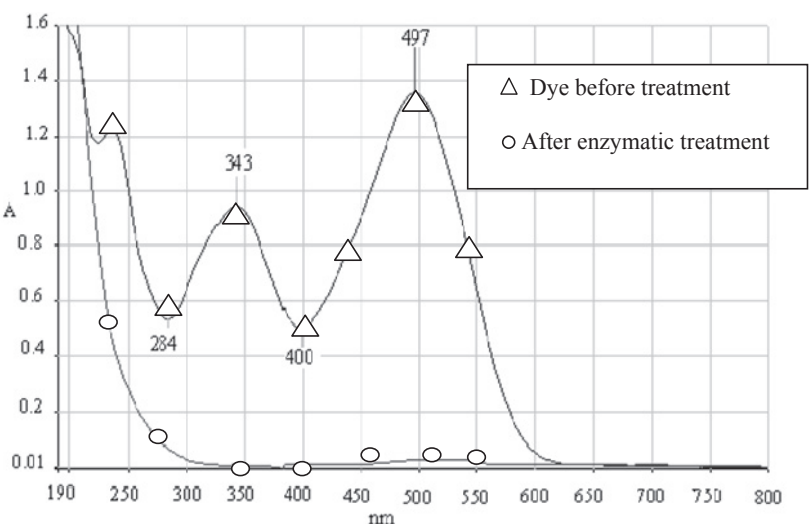

Fig. 9. UV-Vis spectrum of the Congo Red dye before and after enzymatic treatment.

in Fig. 9. The characteristic peaks of the dye at 343 and 497 nm disappeared, while a broad peak between 450 and 550 nm could be observed after completion of the reaction (Torres *et al.*, 2003).

The FTIR absorbance spectrum of CR had an absorbance band at 1446.5 cm^{-1} which can be attributed to the azo group (Fig. 10b), in agreement with Busignana and Cogrossi (1964). Indeed, these authors examined 43 azo and di-azo derivatives and suggested that the absorption band of –N=N– is in the area 1400–1450 cm^{-1}. These peaks were absent from the spectra of the precipitated product after enzymatic treatment (Fig. 10a). In the region from 1600 to 1650 cm^{-1}, there was the appearance of a new band at 1647.1 cm^{-1} in the spectrum of the precipitated product which could be attributed to an R-NH_2 group. The azo function in CR had thus been

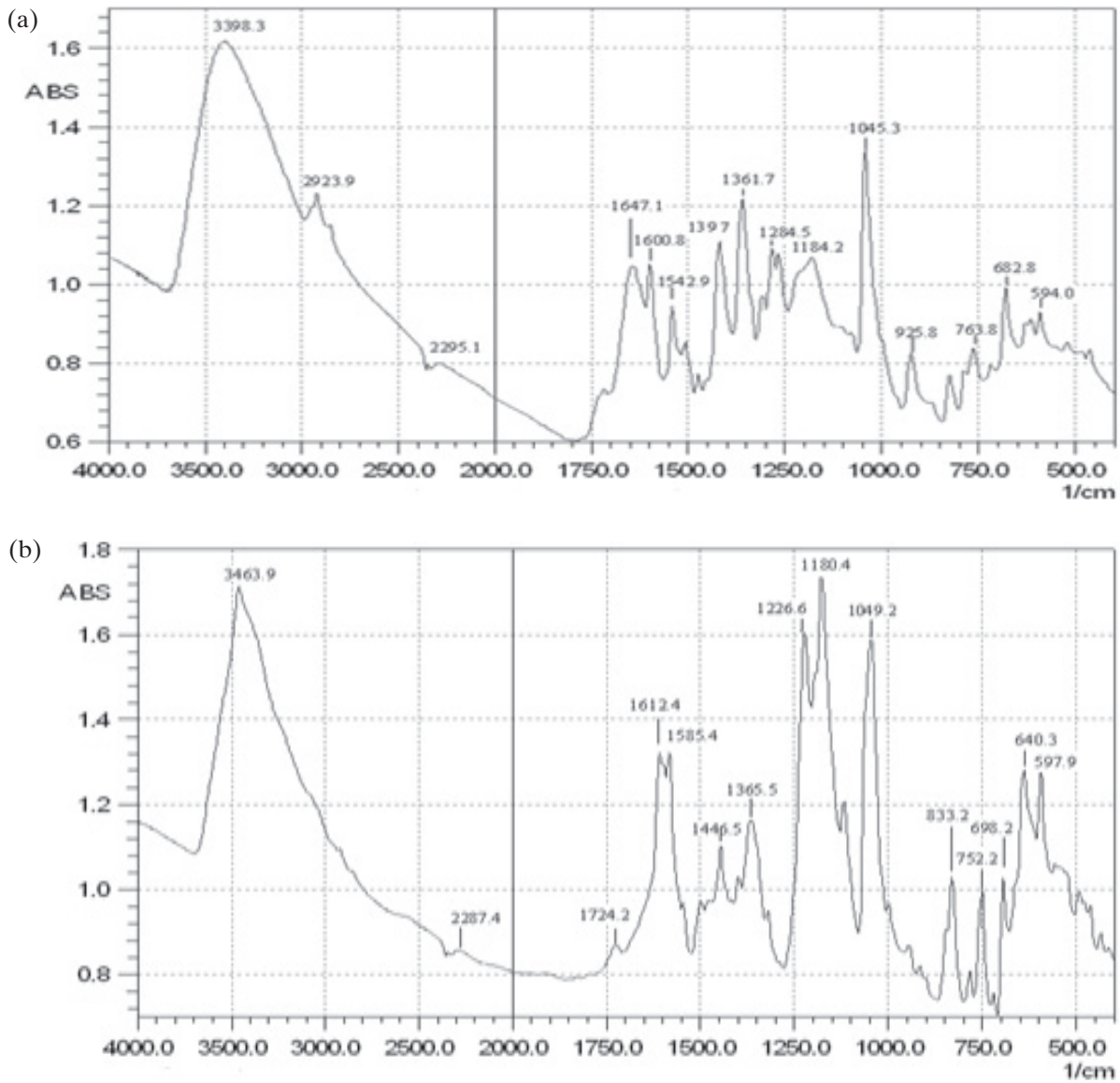

Fig. 10. FTIR spectra of (a) the precipitated product obtained after enzymatic treatment and (b) Congo Red.

replaced by an amide bond in the course of discolouration. A polymerization reaction was probably the explanation. Full analysis by more sophisticated methods such as mass or NMR spectrometry should help in the identification of the nature of the precipitate.

Conclusions

Discolouration of CR using a peroxidase preparation from turnip (*Brassica rapa*) was investigated. The electron-withdrawing nature of the azo linkages obstructs the susceptibility of azo dye molecules to oxidative reactions. Only specialized azo dye-reducing enzymes have been found to degrade azo dyes. The results of this study proved that the use of an enzymatic treatment process is a viable approach for the degradation of azo dyes from aqueous solutions. The optimized discolouration reaction is completed within less than half an hour and non-toxic products are formed (data not shown), including an easily removable precipitate and a dye-free supernatant.

Modeling the kinetics of the enzymatic process and studying the effect of immobilization on the overall rate and discolouration yield are under development in order to prove the feasibility of the procedure for subsequent development on an industrial scale.

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