

Decolourization of Direct Blue 2 by peroxidases obtained from an industrial soybean waste

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ABSTRACT

In this work the decolourization of Direct Blue 2 dye (DB2) using an industrial soybean waste as a source of peroxidases was studied. Temperature, pH, amount of H₂O₂ and concentration of dye were evaluated to determine the maximum catalytic activity of the enzyme during the dye degradation process. It was observed that a temperature of 40°C, a pH of 5 and a concentration of 40 mg/L for the dye in aqueous phase, play a significant role in the overall enzyme-mediated reaction. The maximum decolourization efficiency achieved under optimal conditions was 70% ± 4%. HPLC studies were carried out to confirm dye degradation and analyse the intermediate metabolites. The oxidation products quantified during the reaction were benzidine and 4 aminobiphenyl. Also, an increase in toxicity, determined by *Vibrio fischeri*, was observed after the enzymatic oxidation of the dye. Results suggest that the oxidation of DB2 with peroxidases can be recommended as a pretreatment step before a conventional treatment process.

Keywords: decolourization, Direct Blue 2, industrial waste, soybean peroxidase, oxidation, toxicity

INTRODUCTION

The textile industry is one of the major sources of water pollution due to the volume and composition of effluents produced, which are typically characterized as having a high temperature, pH and colour (Srinivasan and Viraraghavan, 2010; Kumar et al., 2007). The environmental impact caused by this industrial sector is varied, but colour is the first sign of contamination observed in effluents, with concentrations as high as 1 g/L (Kaushik and Malik, 2009). Azo dyes constitute the most important family of industrial dyes. They are recalcitrant and xenobiotic compounds characterized by the presence of one or more azo groups (-N=N-), that make them extremely difficult to eliminate by biodegradative processes (Melgoza and Buitrón, 2004). It has been reported that many of these dyes generate acute and chronic toxicity in ecosystems (Dallago et al., 2005). It is well known that the amount of dye wasted in the effluents depends on the application type used, varying from 2% to 50%, which is then discharged (in the absence of adequate previous treatment) into water bodies (Dos Santos et al., 2007).

Elimination of azo dyes from industrial wastewater is an important issue in wastewater treatment. It has been demonstrated that a single process is not enough for the efficient degradation and mineralization of the dyes and their byproducts (Supaka et al., 2004). Biological processes have been considered as an effective alternative for the

treatment of coloured effluents (Rai et al., 2005), but the need for acclimation, high hydraulic residence times, and the recalcitrant nature of some dyes, as well as the use of additives that improve the fixation of the dyes, constitute the main limitations encountered for stable operation (Hai et al., 2007). Physical, chemical and advanced oxidation processes, such as ozonation, Fenton's reagents and photo-Fenton, have shown good capacity to decolourize these effluents by chemical oxidation, but high investment and operating costs have limited their application (Verma et al., 2012; Prato and Buitrón, 2012; Gutowska et al., 2007).

Recently, studies have been successfully conducted using enzymes with peroxidase activity in the oxidative degradation of coloured compounds, when this cannot be achieved using conventional treatment processes (Matto and Husain, 2007; Mohan et al., 2005). Enzymes with peroxidase activity can specifically react with organic pollutants and remove them by precipitation or the cleavage of the aromatic ring structure, transforming them into other products (Husain, 2010). The catalytic action of enzymes is generally very efficient and selective due to their higher reaction rates and greater stereo-specificity. They can catalyse reactions at relatively low temperature and in the entire aqueous phase pH range, and with lower probability of inhibition caused by substances that might be toxic to microorganisms, and lower cost and retention time, making this process more attractive than conventional treatment methods (Kalsoom et al., 2013; Boscolo et al., 2006).

Peroxidases that have been used for treatment of dyes include horseradish peroxidase (HRP), lignin peroxidase (LiP), manganese peroxidase (MnP) and a number of the other peroxidases obtained mainly from plants, vegetables and microorganisms. HRP is the most often used, due to its ability

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to efficiently cleave aromatic compounds in the presence of peroxide, despite the high costs of its production (Boucherit et al., 2013; Neves et al., 2012; Mohan et al., 2005). Several investigations have reported the use of peroxidases for the removal of azo dyes in aqueous phase, obtaining percentages of decolourization ranging from 50 to 90% using different operating conditions (Nouren and Nawaz, 2015; Silva et al., 2012; Marchis et al., 2011).

In the literature several studies employing soybean peroxidases (SBP) for the decolouration of azo dyes can be found. Calza et al. (2016), applied an enzymatic and photocatalytic treatment with SBP for the removal of three common dyes (Orange I, Orange II, and Methyl Orange), as well as the anticonvulsant drug carbamazepine, from aqueous solutions. SBP was extracted from hulls of fresh soybean (*Glycine max*) seeds and successively purified and immobilized on silica monoliths. All the dyes were completely removed within 2 h, in the presence of immobilized SBP. Chiong et al. (2016) obtained soybean peroxidase and *Luffa acutangula* (luffa) peroxidase, extracted from bio-wastes of soybean hulls and luffa skin peels, respectively, for enzymatic degradation of azo dye methyl orange from liquid effluents. SBP demonstrated a maximum dye decolourization efficiency of 81.4% at 30 mg/L. Ali et al. (2013), investigated the enzymatic degradation of an azo dye (Crystal Ponceau 6R, CP6R) using commercially available SBP enzyme. Under optimized conditions, 40 mg/L dye solution was completely degraded in 1 min in the presence of H₂O₂ and a redox mediator. Dye degradation was also confirmed using HPLC and TOC analyses, which showed that most of the dye was mineralized in the process. Silva et al. (2012) evaluated the potential use of soybean peroxidase in the decolourization of reactive textile dye Remazol Brilliant Blue R (RBBR) and its synthetic effluent. The maximum removal of RBBR (86%) was obtained after 13 min of reaction and at an RBBR concentration of 40 mg/L. Kalsoom et al. (2013), used soybean peroxidase to efficiently degrade Trypan Blue, a diazo dye. The soybean peroxidase (SBP) used in this study was obtained from a commercial supplier and was used as supplied. Studies carried out at different concentrations of the dye (10, 20, 40 and 80 mg/L) yielded a maximum decolourisation efficiency of 90% in the best operating conditions.

Despite the variety of sources of peroxidases, to the best of our knowledge, there are no previous reports related to the recovery of peroxidases from an industrial waste and its application for colour removal. In this work, we present results obtained from a partially purified extract from an industrial soybean waste (okara) and its application in the decolourization of the Direct Blue 2 dye (DB2), widely used in the textile industry for dyeing denim. Effects of different parameters like pH, temperature, reaction time, H₂O₂ dosage and dye concentration were tested in order to optimize the dye degradation. The toxicity before and after the enzymatic treatment was evaluated by *Vibrio fischeri* as a bio-indicator. To identify the principal products or intermediates of the dye oxidation HPLC was utilized.

MATERIALS AND METHODS

Crude extract

The crude extract was prepared from waste (okara) generated by the processing of soybeans for the production of a commercial juice. 200 g of okara were homogenized in 2 L of deionized water for 15 h at 4°C. Then the mixture was

centrifuged at 15 000 × g for 20 min at 4°C (Thermo Scientific Sorval Lynx 4000). The supernatant was dialyzed using 12 kD membrane with a buffer of sodium acetate/acetic acid (1/50), up to a 10 mM concentration at pH 4.5 and 4°C. The dialyzed enzyme extract was stored (4°C) and used for further purification.

Peroxidase activity and protein measurements

The peroxidase activity was determined by the 4-aminoantipyrine method (Bhunja et al., 2001). This colorimetric estimation uses phenol and H₂O₂ as substrates and 4-aminoantipyrine (Am-NH₂) as chromogen. The assay was performed at 25°C; the reaction mixture contained phosphate buffer (0.1 M, pH 7.4), phenol (0.2 M), H₂O₂ (2 × 10⁻³ M), 4-aminoantipyrine (4.8 × 10⁻² M) and enzymatic extract. The consumption rate was estimated measuring the absorbance of coloured product at 510 nm using a UV-vis spectrophotometer (Agilent Technologies Cary 60). A parallel control containing all the ingredients of the assay, except the enzyme, was used as a blank and those without substrate were used as a control. One unit of peroxidase activity (U) represents the amount of enzyme catalysing the oxidation of 1 μmol of substrate during 1 min.

Total protein concentration was measured with the Bio-Rad Bradford protein estimation kit using the microassay procedure with bovine serum albumin as standard protein (Agostini et al., 1997).

Purification of peroxidase

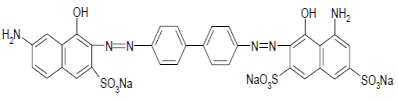
The partial peroxidase purification was conducted by ion exchange chromatography (IEC) using a column of DEAE53-cellulose (2.5 × 15 cm) (Himmelhoch, 1971). The matrix was equilibrated with 0.2 mM and 0.01 mM phosphate buffer (pH 7.4 and 4°C). 700 mL of crude extract were fed to the column at a continuous flow rate of 3 mL/min. The bound protein was eluted using 100 mL of 1 M NaCl. 20 fractions of 5 mL were collected throughout the elution. The fractions were also analysed using UV-visible spectroscopy at 280 nm; enzyme activity and protein concentration were analysed with the previously described methodology. Fractions containing peroxidase activity were then stored at 4°C until use.

Dye and decolourization studies

The dye used in this research was DB2 (Direct Blue 2) donated by a textile industry in México. Some of the chemical properties as well as the structure are presented in Table 1. The aqueous solution of dye was prepared before experiments by dissolving the necessary amount of dye in deionized water.

Decolourization studies were performed on a digital hotplate/stirrer with 9 sites (Dataplate Serie 730 PMC), using 50 mL Erlenmeyer flasks with a working volume of 30 mL. The reaction mixture was carried out using a magnetic stirrer at 150 r/min. A sample was taken each 10 min and then centrifuged at 3 800 × g for 5 min to estimate the residual dye concentration in the aqueous phase. Decolourization percentage of the dye was assessed by changes in absorbance at 576 nm, using a UV-vis spectrophotometer (Perkin-Elmer UV-25 USA). Dye removal percentage was calculated by:

$$\text{Percentage of decolourization: } \left[1 - \frac{C_t}{C_0} \right] \times 100 \quad (1)$$

TABLE 1 Characteristics of DB2	
Name	Direct Blue 2
Colour index	22590
Molecular formula	$C_{32}H_{21}N_6Na_3O_{11}S_3$
Molecular weight	830.71 g/mol
Water solubility	45 g/L (25°C)
Molecular structure	

Where, C_i is the initial concentration of the sample and C_t is the concentration at a given reaction time.

Subsequent series of experiments were performed varying pH (from 3.5 to 7.5), temperature (from 25 to 50°C), H_2O_2 concentration (10 to 500 $\mu\text{mol/L}$) and dye concentration (from 10 to 60 mg/L) to determinate the best conditions for dye removal.

Control experiments for each test were carried out using medium with dye and without enzyme. The experiments were performed in triplicate, and mean and standard deviation were calculated accordingly.

HPLC

The HPLC analyses were performed using a liquid chromatographic system (Thermo Surveyor, auto sampler and diode array detector-DAD). A column C18-Ar ACE (150 \times 4.5 mm) was used for chromatographic separation. The sample was filtered through membranes of 0.2 μm , before injection into the chromatograph. A volume of 30 μL of the sample was injected; methanol/acetonitrile/phosphoric acid 0.1% was used as mobile phase at a flow rate 1 mL/min.

Toxicity test

The Microtox toxicity assays were carried out with *Vibrio fischeri* (Microtox Azure Ambiental). The reagent is a lyophilized preparation from a specially selected strain of the marine bacteria *Vibrio fischeri* (formerly known as *Photobacterium phosphoreum*, NRRL number B-11177). Phenol was used as the reference compound in the toxicity assays (100 mg/L phenol; TU = 3; EC_{50} = 23 mg/L). The toxicity assays were conducted on samples at the beginning and end of the

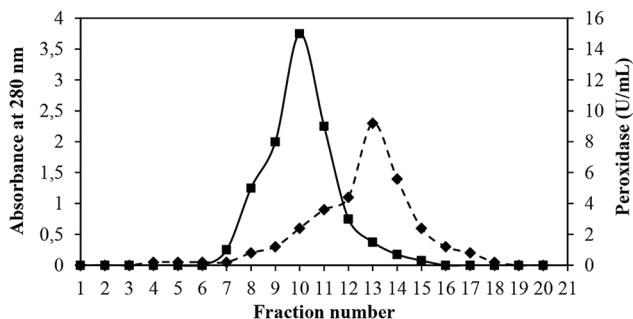


Figure 1

Anion-exchange chromatography of enzymatic crude: (■) peroxidase U/mL, (◆) absorbance at 280 nm

tests to obtain the EC_{50} . Toxicity was expressed in toxicity units (TU), where $TU = 100/EC_{50}$.

RESULTS AND DISCUSSION

Purification of peroxidase

Peroxidase activity of the crude extract was 2.8 U/mL, which is in the range of the reported values obtained from the extract of horseradish root (Gholami et al., 2011; Mohan et al., 2005), ranging from 2.36 to 2.94 U/mL. However, experimental data from this study showed that when partial purification by chromatography using a column of DEAE53-cellulose is applied, the peroxidase activity can be increased to 15 ± 5 U/mL.

Using IEC as a primary purification step increased the degree of enzyme purification with an overall recovery of 45% and specific activity of 22.7 U/mg, obtaining a value of $RZ(Abs_{403nm}/Abs_{280nm}) = 1.1$ for the fraction with the highest enzymatic activity. Figure 1 shows the elution profile of the enzyme in the DEAE53-cellulose column. Similar results have been reported in the literature where peroxidases were obtained from plants and vegetables by conventional methods. Shaffiqu et al. (2002) found peroxidases with high enzymatic activity using plants *I. palmata* and *S. spontaneum* collected in Trivandrum, India; the crude extract was purified by ion exchange and gel chromatography to obtain specific activities of 34.7 U/mg of protein with a purification factor of 77.8%. Gholami et al. (2011) obtained an extract from horseradish root of a local plant of Iran with enzymatic activities of 2.36 U/mL, employing ammonium sulfate precipitations as a first-step partial purification to increase peroxidase activity 18.5 U/mL. Boucherit et al. (2013) used local zucchini of Algeria to obtain peroxidases using the acetone precipitation method, obtaining enzymatic activities of 2.19 U/mL and a purification factor of 1.34.

Contact time effect

The decolourization efficiency of DB2 as a function of the enzyme contact time was determined by employing the enzyme at the highest catalytic activity. DB2 was used as the substrate. The enzymatic reactions were performed at 25°C using 60 mg DB2/L and maintaining pH at 6.5, 200 $\mu\text{mol/L}$ of H_2O_2 , extracted enzyme and 240 min of reaction time. Dye removal attained a maximal value of $50\% \pm 1.95$. After 200 min no more removal was observed and 200 min was used as the reaction time for subsequent experiments (Fig. 2). Similar decolourization percentages have been observed in

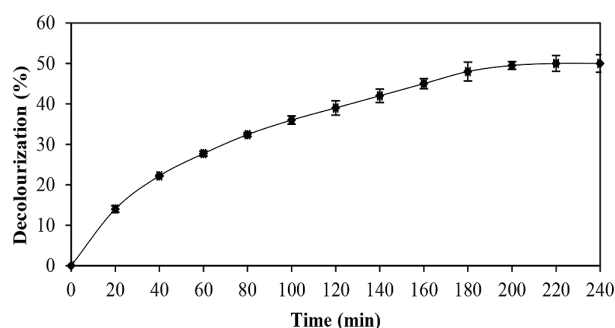


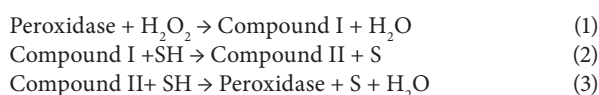
Figure 2

The impact of reaction time on the decolourization of DB2 in presence of peroxidase enzyme

other studies (Silva et al., 2012; Mohan et al., 2005; Shaffiqu et al., 2002). It has been reported that the decolourization time showed a relationship with the chemical structure of the dyes. In general, colourants with simple structures and low molecular weights usually exhibited higher rates of dye removal, whereas dye removal is less effective with highly substituted and high molecular weight dyes (Chen et al. 2003).

Effect of pH and temperature

Studies of pH and temperature were carried out keeping constant the concentration of DB2, reaction time, enzyme and peroxide, at 60 mg/L, 200 min, 15 U/mL and 200 μ mol/L respectively. The pH was varied between 3.0 to 7.5 and the temperature from 25 to 60°C. The enzymatic activity of peroxidase enzyme presented a maximal value at pH 5.5 (Fig. 3A). The highest dye removal rate was found at pH 5.5, reaching up to 64% \pm 1.5. Previous studies have reported that the optimum pH for the decolourization of the diazo dyes by soybean peroxidases is 4 (Kalsoom et al., 2013). Also, for the decolourization of Remazol Blue by HRP, a pH of 2.5 was proposed (Bhunja et al., 2001). This dependence on pH can be explained by the mechanism of the catalytic cycle of peroxidases (Muhammad and Salman, 2012), that involves the formation of two intermediate compounds, namely Compound I and Compound II, where SH indicates a generic substrate, according to the following reactions:



In acidic conditions and in the presence of hydrogen bonding between heme/H₂O₂, distal histidine and arginine side chain, the formation of Compound I is favoured. The rates of the substrate oxidation processes in Steps 2 and 3 have been found to be dependent on the protonated state of Compound I (Laurenti et al. 2003), explaining why soybean peroxidases showed better degradation performance in acidic media.

It was found that when increasing the temperature from 25 to 40°C, the decolourization percentages increased to 56% (Fig. 3B). Nevertheless, beyond 40°C no more improvement was observed and at 60°C only 43% of decolourization was reached. Similar data have been described previously. In the degradation of direct azo by *Curcubita pepo* peroxidases the optimal temperature was 40°C (Boucherit et al., 2013), and 30–35°C for the treatment of Tripan blue by SBP. It has been reported that soybean peroxidase is a thermally stable enzyme at 90.5°C and pH 8 (Kalsoom et al., 2013).

Effect of H₂O₂ concentration

Hydrogen peroxide acts as a co-substrate to activate the enzymatic activity of peroxidase. That promotes the catalytic cycle of peroxidases, where initially the native enzyme is oxidized to form an intermediate enzyme, which accepts the aromatic compounds to oxidize them to a free radical form. The excess of this reagent in the reaction inhibits the enzyme activity and, when added in low quantities, limits the reaction rate (Silva et al., 2012; Wu et al., 1994). The H₂O₂ doses varied between 50 and 300 μ mol/L, and it was found that 200 μ mol H₂O₂ /L promoted the highest enzyme activity and achieving decolourization percentage of 57% \pm 2% (Fig. 3C). However, no greater differences in the enzymatic activity were found with

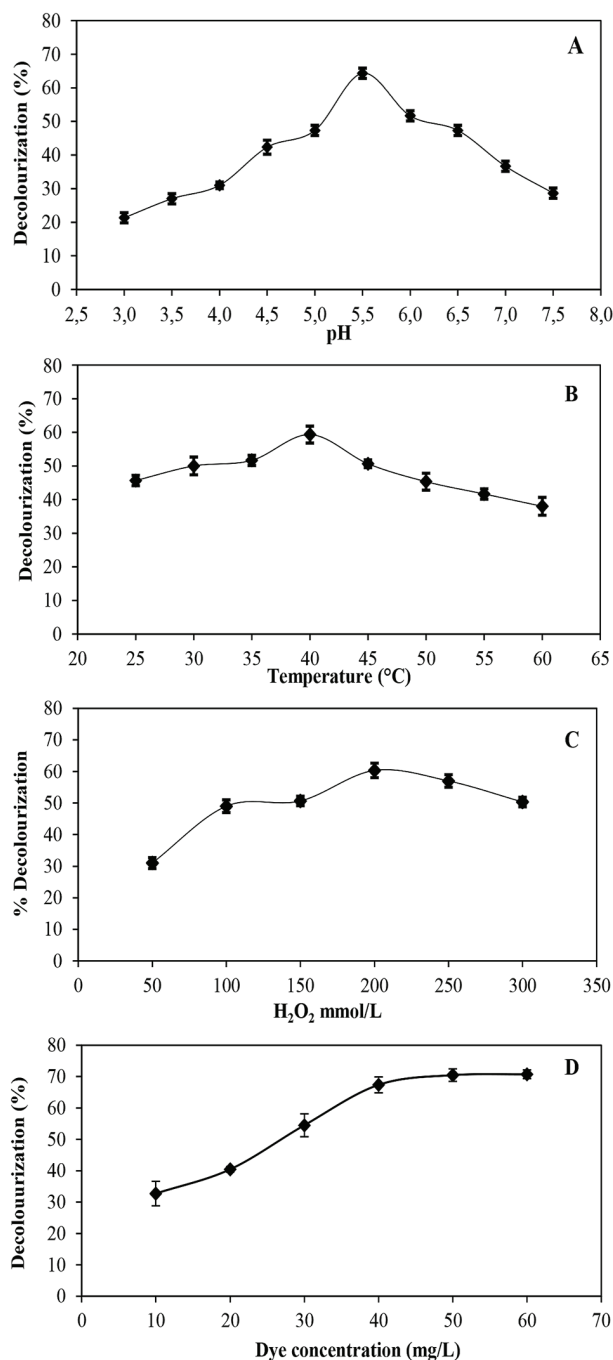


Figure 3
Effect of (A) pH (B) Temperature (°C) (C) H₂O₂ (mmol/L) and (D) Dye concentration (mg/L) on the decolourization of DB2 in presence of peroxidase enzyme

the 100, 150, 250 and 300 μ mol/L doses, having an average decolourization percentage of 51.7% \pm 1.92. Concentrations less than 50 showed a decrease in reaction rate. Similar results have been reported employing 100 μ mol/L of H₂O₂, achieving decolourization of 57% by turnip peroxidase (Silva et al., 2012).

Effect of dye concentration

To evaluate the effect of the dye concentration (from 10 to 60 mg/L) on decolourization percentage, pH (5.5),

temperature (40°C), enzyme concentration (15 U/mL), peroxide concentration (200 µmol/L) and reaction time (200 min) were maintained. It was found that from 10 to 30 mg DB2/L, the decolourization percentage increased steadily. The highest percentage of decolourization (70%) was achieved for concentrations higher than 40 mg/L (Fig. 3D). Similar results were obtained with other peroxidases where the initial decolourization rate increased with increasing dye concentration (Boucherit et al., 2013; Silva et al., 2012; Alam et al., 2009). The concentration of the substrate present in the aqueous phase has a significant influence on any enzyme-mediated reaction. If the enzyme concentration is kept constant and the substrate concentration is gradually increased, the reaction will increase until it reaches its maximum enzyme activity; after obtaining the equilibrium state any further addition of the substrate will not change the rate of reaction (Mohan et al., 2005).

The UV-visible absorbance spectra of 40 mg DB2/L, before and after enzymatic treatment under optimum conditions, are shown in Fig. 4. The spectrum exhibits a main peak with a maximum absorbance at 576 nm. The diminution in the peaks of the dye took place due to the removal of dyes by precipitation or the cleavage of the aromatic ring structure transforming them into other products after treatment with partially purified peroxidase. It could be supposed that azo bonds (–N=N–) of characteristic conjugated chromophores in azo dye molecules were broken (Wang et al., 2009). However, some extra peaks appeared in the treated solution at 212 and 265 nm, probably resulting from the absorbance of metabolites or degraded fragments of the dye molecule (Boucherit et al., 2013).

In our study the enzyme concentration was not varied. The maximum enzymatic activity obtained in this work was 15 U/mL, achieving 70% decolourization. Furthermore, in previous studies it was found that an increase in decolourization percentage of only 4% was achieved when the enzymatic activity was increased from 14.88 to 29 U/mL (Boucherit et al., 2013; De Souza et al., 2007).

Metabolic by-products

The samples were analysed with the HPLC technique to identify the decolourization products in the enzymatic treatment. Control experiments were carried out using dye without treatment. The peak with retention time of 4.2 min was identified as the DB2. After enzymatic treatment the DB2 was reduced and two new peaks corresponding to benzidine and 4-aminobiphenyl (retention times of 1.5 and 7.2 min)

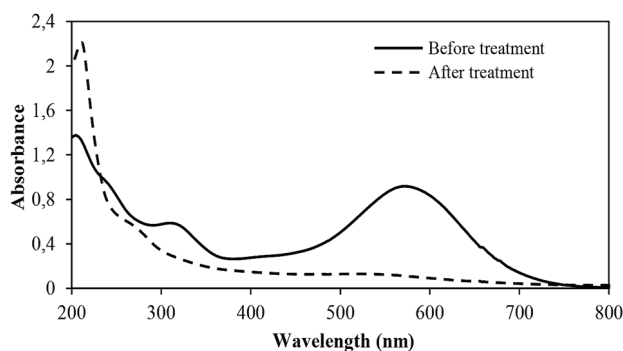


Figure 4

UV-vis spectra of DB2 at 40 mg/L before and after enzymatic treatment

were observed. These compounds have been reported as DB2 by-products (Bafana et al., 2007; Golka et al., 2004). According to the stoichiometry of the reaction, only 20% of benzidine and 10% of 4-aminobiphenyl were recovered from the degradation of DB2 (Fig. 5). That may be due to the parallel production of other compounds, which are generated by different pathways of degradation or by the natural degradation of intermediaries. Alternatively, these products are not final products, so continue to degrade; or there are other ways of generating other or more degradation products, which were not studied in this research.

Toxicity test

A toxicity study of the effluent after the enzymatic treatment was carried out to evaluate whether the reaction products would be more toxic than the original dye. Controls such as enzyme, peroxide and acids were tested to take into account their influence. It was concluded that those compounds do not increase the toxicity. Table 2 shows the toxicity results obtained using *Vibrio fischeri* at 40, 50 and 60 mg DB2/L. The toxicity for the non-treated dye was 4.4 TU, but after treatment the toxicity increased to 17. The toxicity increase can be attributed to the formation of toxic metabolites as benzidine and 4-aminobiphenyl. Silva et al. (2012) reported an increase in acute toxicity after enzymatic treatment of Reactive Blue 21 using *L. sitiva*. They attributed the increase of the toxicity to the formation of metabolites that are more toxic than the parent molecule. The toxicity of this effluent can be eliminated by a combination of biological processes in which the microbial consortia could degrade the byproducts formed in the enzymatic step.

TABLE 2
Acute toxicity test with *Vibrio fischeri*, before and after 200 min of enzymatic treatment at concentrations of 40, 50 and 60 mg/L

Sample	CE ₅₀ (%)	TU
Dye	22.29	4.48
Effluent 40 mg/L	6.24	16.02
Effluent 50 mg/L	5.46	18.31
Effluent 60 mg/L	5.6	17.85

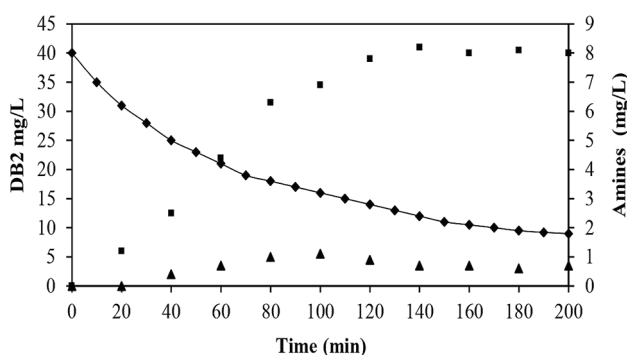


Figure 5

Profile of enzymatic oxidation of DB2 and by-products formation: (◆) DB2; (■) Benzidine; (▲) 4-aminobiphenyl

CONCLUSION

The experimental results obtained in this research showed the effectiveness of using an industrial waste from soybean to obtain enzymes with peroxidase activity, and its application for the decolourization of DB2. In the experimental tests the data reveals that the performance of a reaction catalysed by partially purified peroxidase for dye removal depends upon the reaction time, temperature, pH and dye concentration, with all of these being key factors that affect the rate of oxidation. With the best operating conditions, it was possible to achieve 70% ± 4% decolourization for dye concentrations higher than 40 mg/L (pH 5.5, 40°C, 200 µmol H₂O₂/L). Benzidine and 4-aminobiphenyl were identified as the main by-products of the enzymatic degradation of DB2. An increase in toxicity after enzymatic treatment of the DB2 was observed and was attributed to the formation of aromatic amines. Based on these findings, it is concluded that the enzymatic treatment can be used as a previous step (pre-treatment) to conventional processes, representing an important step to facilitate the mineralization of textile dyes.

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