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RESEARCH PAPER

Oxidation and Removal of Industrial Textile Dyes by a Novel Peroxidase Extracted from Post-harvest Lentil (*Lens culinaris* L.) Stubble

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Abstract The degradation and removal of a series of dyes used in the textile industry for polyester/wool (PES/WO) blends and present in effluents, such as Green, Ash-Grey, Black, Navy Blue, Red and Yellow Domalan, and Orange and Red Bemacid, by catalytic action, in the presence of H_2O_2, of extracts of a novel peroxidase from post-harvest lentil stubble was investigated. The extracts of this peroxidase (LSP) were effective in degrading these last-generation textile dyes, especially Green Domalan, Orange Bemacid, Grey and Black Domalan. A sensitivity study was carried out for Green Domalan biodegradation to determine the effects of process parameters such as pH, H_2O_2, enzyme and dye concentrations, contact and centrifugation times, and temperature. Standard ecotoxicity studies performed with *Vibrio fischeri* revealed that the dye solutions treated with peroxidase and H_2O_2 were less ecotoxic than the untreated ones.

Keywords: azo, anthraquinone, textile, dyes, peroxidase, oxidation, decolorization, ecotoxicity test

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1. Introduction

Most synthetic industrial dyes are complex aromatic compounds with an azo bond connected to several aromatic structures. Some, however, are polymeric structures containing metals.

It is estimated that there are over 10,000 commercially available dyes and pigments for industrial use, representing an annual consumption of around 7×10^5 tons worldwide [1,2]. However, about 10 ~ 15% of the synthetic dyes produced are discharged into industrial effluents [3], causing environmental problems. Accordingly, in many countries the contamination of water bodies by dyes is an important problem.

The colour of textile effluents is due to the dyes. Synthetic dyes vary in chemical composition, but they share a common feature: they must be highly stable against light and washing and they must be resistant to chemical and microbial attack [4]. Among the chemical classes of dyes, azo dyes, the largest class of these compounds (84% of all dyes used), are considered to be recalcitrant, non-biodegradable by aerobic bacteria, and persistent. The anaerobic process has a major disadvantage in that the reduction of azo dyes generates colourless but potentially mutagenic and carcinogenic aromatic amines [5]. Consequently, the treatment of dye-polluted effluents is considered to be one of the most challenging tasks among the environmental community [6].

Physicochemical methods, such as adsorption by activated carbon, ultrafiltration, coagulation, irradiation, oxidation (with chlorine, hydrogen peroxide, and ozone), reduction, precipitation, electrochemical treatment, and ion-pair extraction are effective in the removal of dyes. However, none of these methods has been found to be very suitable because they are expensive and produce toxic pollutants,

and commonly used methods of chemical precipitation produce large quantities of sludge, which creates problems of disposal [4,7].

Consequently, owing to its highly selective nature, researchers have been focusing their attention on the study of enzymatic pretreatment as a potential and viable alternative to conventional methods. Further, in enzymatic treatment, inhibition by toxic substances is minimal and the process can operate over a broad concentration range of aromatics, with low retention times [8]. Enzymes can act on specific recalcitrant pollutants to remove them by precipitation or transformation to other (innocuous) products and can also change the characteristics of a given waste to render it more amenable to treatment. Compared to chemical catalysts, the catalytic action of enzymes is extremely efficient and selective owing to higher reaction rates, milder reaction conditions, and greater stereospecificity. Enzymes can catalyze reactions at relatively low temperatures and across the whole aqueous pH range. Although much attention has been paid to the use of biocatalysts in several fields, their involvement has only been considered very recently for the resolution of environmental problems [8,9].

Dyes can be removed by means of oxidative enzymes [10-12]. Peroxidases are versatile enzymes that catalyze the oxidation of a large number of aromatic structures through a reaction with hydrogen peroxide, and they are applied in the chemical, environmental, pharmaceutical, and biotechnological industries. Peroxidases can act on specific recalcitrant pollutants either by free radical oxidative polymerization and consequent settling or by transformation into other products [13]. Horseradish peroxidase (HRP) in the presence of H_2O_2 is known to be effective in the removal of a broad spectrum of aromatic compounds (phenols, biphenols, and anilines) and in the degradation and precipitation of industrial dyes [9,11,14-16].

The objectives of the present study were to extract potent peroxidases from agricultural biowastes, in particular from post-harvest fresh lentil leaves, and to use their reaction mechanism for the oxidation/removal of a series of dyes commonly found in the contaminated effluents of textile industries. The study is aimed at checking the capabilities of such peroxidase extracts coupled to H_2O_2 in the oxidation of those textile dyes, in particular of Green Domalan BL. The effects of parameters such as pH, H_2O_2, enzyme and dye concentrations, contact and centrifugation times, the temperature of decolorization, and the detoxification of the dye were investigated.

2. Materials and Methods

The Domalan and Bemacid dyes were a gift from Dyes Alçada y Pereira, LDA (Covilhâ, Portugal). Domalan dyes are manufactured by M. Dohmen GmbH & Co. KG (Korschenbroich, Germany) and Bemacid dyes by BEZEMA AG (Montlingen, Switzerland). The dyes (40 ~ 800 mg/L) were dissolved in 0.02 M acetic acid/acetate, pH 4.0 ~ 4.7, the buffer present in the effluents of this textile industry. The molecular weight of Green Domalan BL by mass spectroscopy was 930 g/mol.

Peroxidase (LSP) was extracted and purified from lentil stubble obtained just after harvesting in the *Armuña* area, near the city of Salamanca (Spain). Green leaves and stems were milled and incubated in distilled water with constant stirring for 22 ~ 24 h at room temperature. The homogenate obtained was vacuum-filtered and centrifuged (10,000 g, 277 K for 20 min). Pigments were extracted by phase separation over 20 ~ 22 h at 277 K after the addition to the supernatant of solid PEG to 14% (w/v) and solid ammonium sulphate to 10% (w/v). Two phases were formed after the addition of ammonium sulphate: an upper polymer phase (dark red in colour), which contained pigments, phenols, polyphenols, oxidized phenols and PEG, and a lower aqueous phase (yellow in colour) containing peroxidase. Each phase consisted of 50% of the initial volume. These phases were separated and the aqueous phase, containing peroxidase activity, was centrifuged (10,000 *g*, 277 K, for 15 min). The clear supernatant, containing peroxidase activity, was the final extract used in the experiments of oxidation and removal of industrial textile dyes. To proceed further purification of LSP, this extract was titrated with ammonium sulphate to a conductivity value of 326 mS/cm and was applied on a Phenyl-Sepharose CL-4B column (1.5 × 35 cm) equilibrated with 100 mM phosphate buffer, pH 6.5, with 1.7 M ammonium sulphate, which has the same conductivity as the sample. The enzyme was eluted with 100 mM phosphate buffer, pH 6.5, plus 0.2 M ammonium sulphate at a flow rate of 1 mL/min. 12 mL fractions were collected and those showing peroxidase activity were dialyzed against 10 mM Tris buffer, pH 8.1, with constant stirring for 24 h at 277 K. After being centrifugation (10,000 g, 277 K for 20 min), these fractions were 6 times membrane-concentrated (Amicon, 30 kDa cutoff).

The purity of the LSP was determined by SDS-PAGE as described by Fairbanks *et al.* [17] on a Bio-Rad minigel device using a flat block with a polyacrylamide gradient of 4 ~ 12%. Gels were prefixed and stained using the method of Merril *et al.* [18]. Purity was also determined by UV-Visible spectrophotometry ($RZ = A_{403}/A_{280} = 2.5 \pm 0.1$). Protein concentrations were determined by the Bradford assay [19].

LSP was partially purified with a high yield from post-harvest lentil stubbles. The purification steps and their

Table 1. Purification steps of LSP

Procedure	Volume (mL)	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification	Yield (%)
Homogenate	1,040	9,161	64,480	7	1	100
PEG + $(NH_4)_2SO_4$	490	1,332	63,700	48	7	98.8
Phenyl-Sepharose	10	17	17,650	1,038	147	27.4

efficiencies are summarized in Table 1. Purified peroxidase migrated in SDS-PAGE as three main bands corresponding to molecular weights of 52, 35, and 18 kDa. This molecular weight range is characteristic of some other plant peroxidases [20-22].

One unit of peroxidase activity (U) was defined as the amount capable of oxidizing 1 μmol of guayacol (2-methoxyphenol) per minute under standard conditions. Peroxidase activity was assessed with a colorimetric method using H_2O_2 as substrate and guayacol as chromogenic substrate. The assay was performed at 20ºC by adding 20 μL of the enzyme solution to 2.0 mL of 20 mM $H_2PO_4^-/HPO_4^{2-}$, pH 6.0, containing 4.9 mM H_2O_2 and 18 mM guayacol. The rate of H_2O_2 consumption was estimated by measuring the absorbance of a coloured product at 470 nm, using a molar absorptivity of 5,200/M/cm [23]. The extract from lentil stubble was found to contain a peroxidase activity of 102 U/mL.

Quantitative estimation of the dyes in the aqueous phase was carried out by colorimetry, scanning the absorbance spectrum at wavelengths between 350 and 700 nm with a Beckman DU-7 UV/VIS spectrophotometer. For the Green Domalan BL dye solution (76 mg/L), the absorbance maximum was obtained at λ_{max} = 600 nm (Fig. 1, open circle symbols). At this wavelength, the corresponding

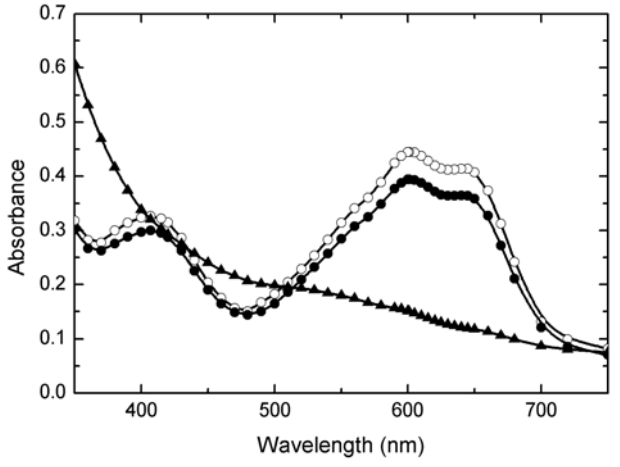

Fig. 1. Electronic spectra of Green Domalan BL (76 mg/L) in 20 mM acetic acid/acetate, pH 4.0, before (○) and after being incubated for 24 h with H_2O_2 (10 mM) (●) and with peroxidase (5.65 U/mL) and H_2O_2 (10 mM) (▲). The decolorization yield in 24 h for the peroxidase process was 79.7%.

Beer-Lambert correlation between absorbance and dye concentration gave a molar absorptivity of 8.6 $mM^{-1}cm^{-1}$, which was used to estimate the dye concentration. After the enzymatic treatment, the dye samples were centrifuged and the supernatants were assayed for residual dye concentrations.

Experiments were conducted to assess dye removal from the aqueous solution by enzyme activity in order to determine the equilibrium time required for dye removal. The experiments were carried out at a constant temperature (21ºC) but varying the process parameters, namely pH, dye, peroxidase and H_2O_2 concentrations, and centrifugation and incubation times. Initially, the kinetic runs were carried out at a 76 mg/L dye concentration by keeping the aqueous phase pH at 4.0, 5.65 U/mL peroxidase and 10 mM H_2O_2 constant. The reaction mixtures in the vials were shaken at 100 rpm for the requisite contact times and aliquots of the solution were analyzed for residual dye concentrations in the aqueous phase after centrifugation (10,000 *g*, 2 min, 20ºC).

Currently, the most common bacterial ecotoxicity bioassay is based on monitoring changes in the emissions of natural light from a luminescent bacteria: live *Vibrio fischeri* (*Photobacterium phosphoreum*) [24,25].

In the presence of pollutants, the natural bioluminiscence of *Vibrio fisheri* decreases and toxicity is expressed as the lethal concentration 50 (LC_{50}); *i.e.*, the concentration of the pollutant that causes a 50% reduction in the initial light output [26,27].

Here we measured the ecotoxicity of synthetic textile wastewater samples using the methodology described in reference [28]. For ecotoxicity testing, the sample was salted with 2% NaCl. When performing the test, 2% NaCl and 20% NaCl were required. The main reason for using these salt solutions is to ensure a proper osmotic balance of the bacteria, thus ensuring that the decrease in light emission of bacteria will by due solely to the effect of the pollutants [29]. Then, the sample was incubated at 15ºC for 15 min and, finally, sample measurement was performed in triplicate with a MULTITOX BG-1 luminometer, which gives the LC_{50} values and the inhibition curve of light.

If a sample shows no decrease in light emission at any dilution, it is not considered toxic and there is no value for its LC_{50}. In the case of wastewaters, ecotoxicty units in

equitox m^{-3} are accepted (equitox m^{-3} = (1/ LC_{50}) × 100).

3. Results and Discussion

Horseradish peroxidase is known to degrade phenol and substituted phenols and amines via a free radical oxidative polymerization mechanism [13,30-33]. Depending upon its substrate specificity, horseradish and other plant peroxidases could be applied for the degradation of other recalcitrant organic compounds containing aromatic/phenolic groups [4].

The last generation of Domalan textile dyes are confidential combinations of anthraquinone and azoic acid-dispersed dyes and Bemacid dyes are also confidential azo dyes with 2 sulfonic acid groups (Fig. 2).

Such dyes with complex structures contain several aromatic groups (phenol, aniline), making them possible substrates of lentil stubble peroxidase (LSP). Screening experiments designed to assess dye degradation in the presence of H_2O_2 and LSP gave positive results. The dye solutions were found to be stable upon exposure to H_2O_2 alone and to the enzyme extract alone. Thus, dye precipitation is a result of an H_2O_2-dependent enzymatic reaction, possibly involving free-radical formation followed by polymerization and precipitation.

Initially, a study of the decolorization efficiencies of the enzymatic oxidation of the dyes was carried out by scanning the visible spectra of the reaction mixture, at different times (0 ~ 24 h), of the dye plus 5.65 U/mL peroxidase and 10 mM H_2O_2 in 20 mM acetic acid/acetate buffer, pH 4.0 (see Fig. 1 for Green Domalan BL dye). These repeated electronic spectra showed the optimum wavelength for following the kinetic runs and establishing the decolorization efficiencies of each dye studied (Table 2).

Since the data in Table 2 suggested that the best decolorization performance was achieved with the dye Green Domalan BL, the following objective was to obtain the maximum percentage of degradation of this dye with the minimum input, thereby minimizing process costs. A sensitivity analysis aimed at determining the optimum conditions for Green Domalan BL removal was carried out by varying the following process parameters: pH (from 2 to 9), the reaction (0 ~ 24 h) and centrifugation (2 ~ 10 min) times, peroxidase activity (0 ~ 6.78 U/mL), H_2O_2 (from 0 to 11 mM) and dye (from 20 to 270 mg/L) concentrations.

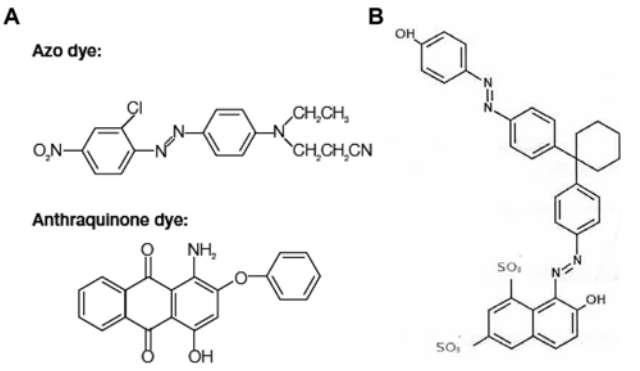

Fig. 2. Azoic and anthraquinone structures of Domalan dyes (A) and the structure of Orange Bemacid dye (B).

Four kinetic runs were performed in order to assess the optimum reaction time required for dye removal: one kinetic run with 20 mL of dye solution (76 mg/L) in 20 mM acetic acid/acetate buffer (pH 4.0) at 25°C; a second one with the dye solution plus 5.65 U/mL peroxidase, a third one with the dye solution plus 10 mM H_2O_2, and a fourth one with the dye solution plus 5.65 U/L peroxidase and 10 mM H_2O_2. At each 10-min time point of the incubation, an aliquot was removed from the mixture and centrifuged for 2 min, after which the residual dye concentration was analyzed (Fig. 3). The runs finished at 24 h, with no evidence or further significant decolorization, except in the third case. From Fig. 3, it may be concluded that the best dye removal capacity was achieved with lentil peroxidase - H_2O_2 coupled oxidation with respect to the

Table 2. Substrate specificity of LSP for the decolorization/degradation of a series of dyes

Dye	λ_{max} (nm)	Time (h)	[Dye] (mg/L)	Abs_0	$Abs_{fin.\ time}$	Decolorization yield (%)
Green Domalan BL	600	24	700	0.4137	0.1211	79.7
Orange Bemacid CMGL	491	72	40	0.6125	0.2140	65.1
Ash-Grey Domalan R	556	24	800	0.6042	0.3571	40.9
Black Domalan HEBD	570	24	700	0.5244	0.3325	36.6
Navy Blue Domalan HEND	590	24	100	1.2952	0.9872	23.8
Red Bemacid F-GS	518	72	110	1.1547	0.8862	23.3
Red Domalan 2BL	541	24	700	0.8541	0.6921	19.0
Red Domalan RL	490	24	150	1.4712	1.3197	10.3
Yellow Domalan 3RL	446	72	150	1.0087	0.9675	4.1

Other experimental conditions as in Fig. 1.

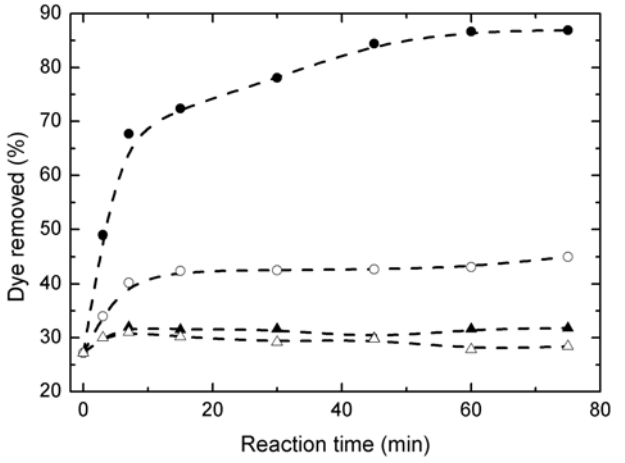

Fig. 3. Time-course for Green Domalan removal mediated by LSP. Dye solution (△), dye incubated with LSP (○), dye incubated with H_2O_2 (▲), and dye incubated with LSP plus H_2O_2 (●). For experimental conditions, see text.

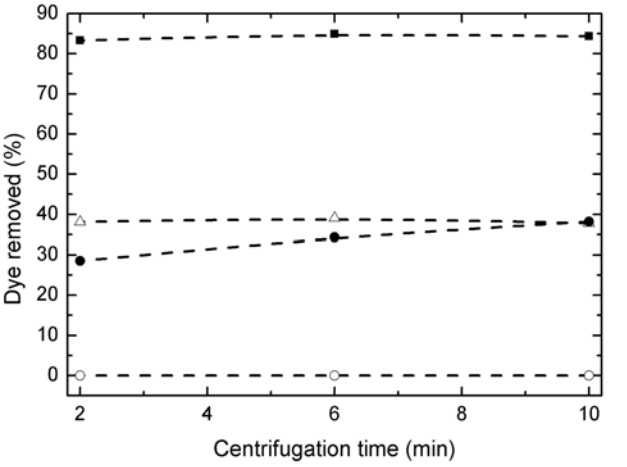

Fig. 4. Effect of centrifugation on LSP-catalyzed Green Domalan removal. Centrifuged (●) and non-centrifuged (○) dye solution, centrifuged (■) and non-centrifuged (△) enzymatic reaction sample. For experimental conditions, see text.

single oxidation of dye by the enzyme alone and H_2O_2 alone. Furthermore, 60 min of reaction time was sufficient for most (87%) of the dye to be removed.

Whereas, at the beginning of the fourth kinetic run of Fig. 3, the standard peroxidase activity was 5.65 U/mL, in the final aliquot of this kinetic run, removed at 24 h, the activity fell to 1.33 U/mL. This 76.5% decrease in peroxidase activity suggests that the reaction product is a polymer which settling, by centrifugation, drags and traps a large proportion of the enzyme molecules, which become immobilized within the polymer matrix. From a practical point of view, this 23.5% of remaining peroxidase activity suggests the possibility of reusing the catalyst in solution for subsequent decolorization processes. Furthermore, it may be assumed that the immobilized peroxidase would be active for catalysing repeated dye removal processes.

Considering that the peroxidase-H_2O_2-driven oxidation of these dyes, with phenol and substituted phenol and amine groups, produces a polymer of high molecular mass [13,30-33] -the polymer that could settle-, one sensitive variable of the process would be the centrifugation time of the reaction mixture to achieve the complete precipitation and removal of the polymer from the solution. A blank sample of 20 mL of dye solution (76 mg/L) in 20 mM acetic acid/acetate buffer (pH 4.0) and a reaction sample of 20 mL of dye (76 mg/L), 10 mM H_2O_2, 5.65 U/mL peroxidase solution in 20 mM acetic/acetate buffer (pH 4.0) were prepared and left for 50 min at 25ºC. Then, aliquots were removed to analyze the residual dye concentration before and after centrifugation at 10,000 rpm for 2, 6, and 10 min. It may be seen from Fig. 4 that 2 min of centrifugation is sufficient for maximum dye removal.

Another interesting observation is that after centrifugation the blank sample achieved about 40% of dye removal and the reaction sample 84%. However, the reaction sample without centrifugation also achieved a 40% dye removal. This suggests that Green Domalan BL removal from aqueous solutions is a combination of processes: A physical process of sedimentation, responsible in this case for about 40% of dye removal, and the peroxidase-H_2O_2-driven oxidation of the dye, producing a polymer that settles spontaneously and is responsible for another 40% of dye removal.

Most enzymes have a characteristic pH range at which their activity is maximal. For all enzymes, the interrelationship of enzymatic activity and pH depends on the acid-base behaviour of the substrate, as well as on other factors such as the reaction environment.

To assess the decolorization activity of LSP as a function of pH, a series of reaction solutions of Green Domalan (76 mg/L), LSP (5.65 U/mL), H_2O_2 (10 mM) in universal buffer (20 mM phosphoric acid, 20 mM boric acid, and 20 mM acetic acid), pH 2 ~ 9, were prepared. The reactions were left to progress for 1 and 24 h at 25ºC, after which aliquots were taken and the dye concentrations in the solution were analyzed after centrifugation for 2 min. A conventional bell-shaped curve with a defined pH (3.3) for maximum activity (87% dye removal) was obtained for aliquots taken at 1 h of reaction time (Fig. 5). However, when the reaction time was 24 h, the dye-removing activity *versus* the pH profile was different, with a maximum plateau range activity (87% dye removal) between pH 3 and 6. This profile, typical of immobilized enzymes [34], also points to the notion that the polymer formed causes the immobilization of LSP, limiting free dye and H^+ diffusion towards the enzyme and disturbing the typical bell-shaped profile of

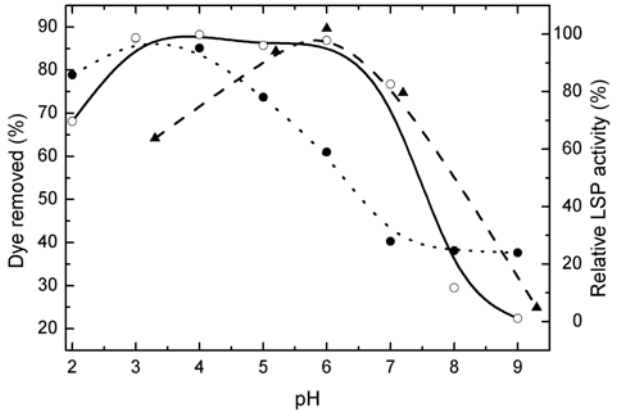

Fig. 5. Effect of pH on LSP catalyzed Green Domalan removal from aqueous solutions. Dye removal activity *versus* pH at 1 h (●) and 24 h (○) reaction. LSP activity towards guayacol *versus* pH (▲).For experimental conditions, see text.

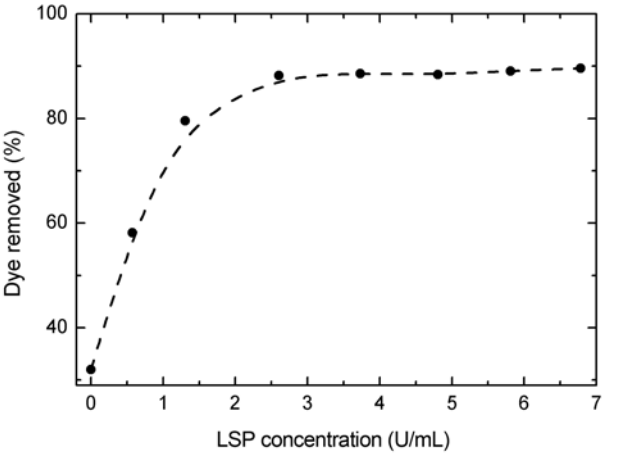

Fig. 7. Effect of LSP concentration on Green Domalan removal activity. See text for experimental conditions.

enzymes in solution (see the profile of peroxidase activity against guayacol *versus* pH in Fig. 5). Furthermore, it should be noted that the pH of industrial textile effluents is around 4.0 and at this pH the dye removal activity of LSP is maximal.

Hydrogen peroxide acts as a co-substrate to activate the enzymatic action of the peroxide radical. In the catalytic cycle of peroxidase, it contributes to the oxidation of the native enzyme to form an enzymatic intermediate that accepts the aromatic compound to carry out its oxidation to a free radical form. Experiments were carried out to find the optimum H_2O_2 concentration required to bring about the removal of dye by varying the H_2O_2 dose from 0.03 to 11 mM in the reaction mixture, keeping all the other experimental conditions constant (76 mg/L dye, 5.65 U/mL LSP, 20 mM acetic acid/acetate buffer (pH 4), 25ºC, 1 h reaction). The results obtained are shown in Fig. 6, where it can be seen that an H_2O_2 concentration of 0.3 mM was sufficient for maximum dye degradation. H_2O_2 concentrations higher than 1 mM inhibited the dye-removing activity of LSP.

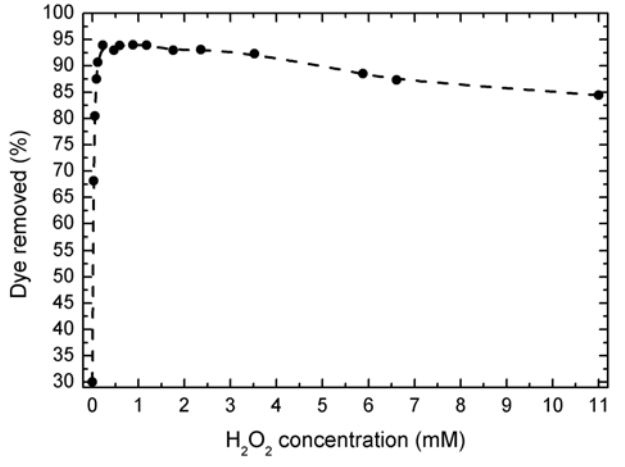

Fig. 6. Effect of H_2O_2 concentration on LSP-catalyzed Green Domalan removal from aqueous solutions. For experimental conditions, see text.

Usually, the removal of an aromatic compound is dependent on the concentration of the enzyme (catalyst) added. There is an optimum relationship between the concentration of enzyme and substrate for achieving maximum activity. The experimental conditions were 76 mg/L dye, 0.3 mM H_2O_2 in 20 mM acetic acid/acetate buffer, pH 4, 25ºC, 60 min reaction time. Within the LSP concentrations evaluated (0.57 ~ 6.78 U/mL), it was observed that when the peroxidase activity was 0.57 U/mL, 58.1% of the dye was decoloured. However, when it was 2.26 U/mL, decolorization reached 88.6% (Fig. 7). The enzyme concentration was found to have a significant influence on the dye removal yield. Optimization of the concentration of enzyme to be used was carried out aiming at high decolorization efficiency with the lowest quantity of enzyme in free form used in the process. Thus, the optimum LSP concentration was considered to be 1.13 U/mL because a high yield of dye removal was achieved (81%). Under the experimental conditions studied, no enzyme deactivation was observed.

The concentration of substrate present in the aqueous phase has a significant influence on all enzyme-mediated reactions. If the enzyme concentration is kept constant and the substrate concentration is gradually increased, the reaction rate will increase until it reaches a maximum. Experiments were carried out at different dye concentrations (2 ~ 270 mg/L), keeping all the other parameters constant (1.13 U/mL LSP, 0.3 mM H_2O_2, 20 mM acetic acid/acetate buffer, pH 4.0, 25ºC, 60 min reaction time). Dye removal was measured as the difference between the dye concentration of the reaction sample after centrifugation and the amount of dye removed by the centrifugation of a dye blank of the same concentration. The

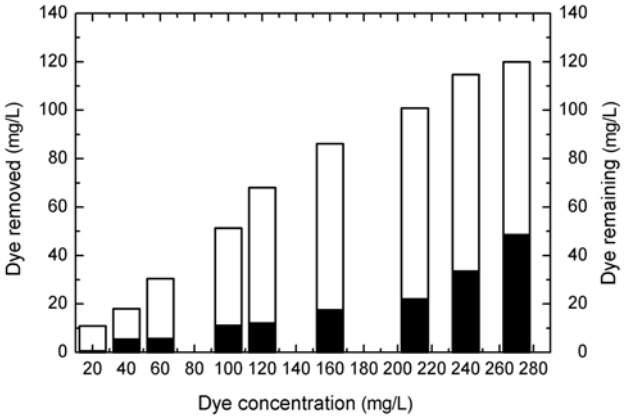

Fig. 8. Effect of dye concentration on LSP-catalyzed Green Domalan removal from aqueous solution. Dye removed (□) and remaining (■). For experimental conditions, see text.

results (Fig. 8) indicated that the increase in dye concentration resulted in greater dye removal, being maximal at 240 mg/L.

Temperature is an important variable in all enzyme kinetics. The effect of the reaction temperature was studied for Green Domalan BL between 18 and 38ºC, keeping all the other parameters constant (20 mM acetic acid/acetate buffer, pH 4.0, 60 min reaction time, 0.3 mM of H_2O_2, 1.13 U/mL LSP, 240 mg/L of dye). The variations in dye removal at different temperatures are shown in Fig. 9.

From the kinetic runs shown in Fig. 9, the slight influence of temperature on the initial reaction rates, but not on the equilibrium concentrations (equilibrium reached at 1 h of reaction time) can be seen. These data point to a standard enthalpy of the process close to zero and an activation energy of 13 kJ/mol. These low values of ΔH^o and E_A suggest that the rate-determining step of the decolorization of Green Domalan by the biocatalytic action of LSP is a physical process of mass transport driven by diffusion and, being a physical process, it is not very influenced by temperature. According to different authors [13,30-33], these peroxidase bleaching processes involve the oxidative polymerization of the dye, and the reaction product is a complex polymer that allows the existence of diffusional limitations to the free access of the reagents to the catalytic centre of the enzyme.

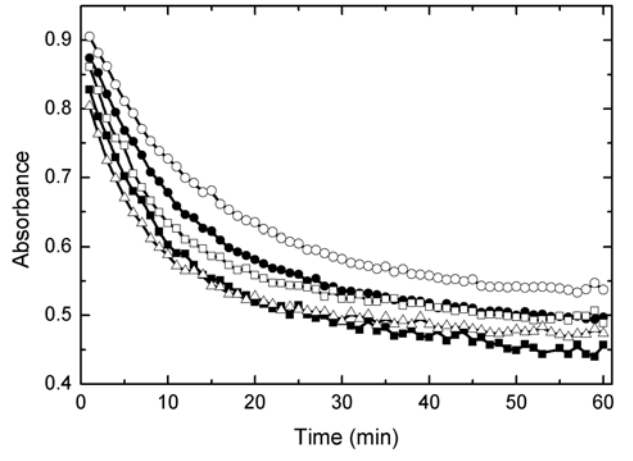

Fig. 9. Kinetic curves of the oxidation/removal of Green Domalan BL by LSP in the presence of H_2O_2 at different temperatures: 18ºC (○), 23ºC (●), 28ºC (■), 33ºC (□), and 38ºC (△). Otherexperimental conditions in text.

Summarizing the optimization studies of the parameters of the Green Domalan decolorization process, the main conclusions of the sensitivity analysis are shown in Table 3.

The response established for the toxicity test with *Vibrio fischeri* was the mean lethal concentration (LC_{50}) at which 50% of the bacteria were killed during 24 h of exposure to the effluent. The toxicity study of the Green Domalan BL effluent before and after the enzymatic (+H_2O_2) treatment for 24 h was carried out to assess whether the reaction products would be more toxic than the effluent itself.

Treatment experiments were carried out on Green Domalan BL in 20 mM acetic acid/acetate, pH 4.0, 2.3 U/mL LSP,

Table 3. Sensitivity study for the determination of the optimum conditions for the performance of lentil stubble peroxidase (LSP) with regard to Green Domalan BL removal from aqueous solutions

Parameters	Range evaluated	Optimized parameters
pH	2.0 ~ 9.0	3.0 ~ 5.0[a]
Reaction time (h)	0 ~ 24	1[b]
Centrifugation time (min)	2 ~ 10	2[c]
Peroxidase activity (U/mL)	0 ~ 6.78	1.13[d]
[H_2O_2] (mM)	0 ~ 11	0.3[e]
[Dye] (mg/L)	20 ~ 270	240[f]
Temperature (ºC)	18 ~ 38	25[g]

Other conditions: (a) 20 mM universal buffer, 76 mg/L dye, 5.65 U/mL LSP, 10 mM H_2O_2, 24 h reaction time, 25ºC. (b) 20 mM acetic acid/acetate, pH 4.0, 76 mg/L dye, 5.65 U/mL LSP, 10 mM H_2O_2, 50 min centrifugation, 25ºC. (c) 20 mM acetic acid/acetate, pH 4.0, 76 mg/L dye, 5.65 U/mL LSP, 10 mM H_2O_2, 1 h reaction, 25ºC. (d) 20 mM acetic acid/acetate, pH 4.0, 76 mg/L dye, 0.3 mM H_2O_2, 2 min centrifugation, 1 h reaction, 25ºC. (e) 20 mM acetic acid/acetate, pH 4.0, 76 mg/L dye, 5.65 U/mL LSP, 2 min centrifugation, 1 h reaction, 25ºC. (f) 20 mM acetic acid/acetate, pH 4.0, 1.13 U/mL LSP, 0.3 mM H_2O_2, 2 min centrifugation, 1 h reaction, 25ºC. (g) 20 mM acetic acid/acetate, pH 4.0, 1.13 U/mL LSP, 0.3 mM of H_2O_2, 240 mg/L dye, 1 h reaction.

Table 4. Toxicity tests with *Vibrio fischeri* after 24 h incubation with Green Domalan before and after enzymatic (+H_2O_2) treatment for 24 h

	LC_{50} (mg/L)	Equitox m^{-3}
Green Domalan BL	0.02	4,789
Green Domalan after enzymatic reaction	1.05	95

See text for other experimental conditions.

0.3 mM H_2O_2, 2 min centrifugation.

The toxicity results obtained (Table 4) reveal a significant decrease in the LC_{50} for the untreated dye sample with respect to the dye sample treated enzymatically.

The LSP enzyme, in soluble form, was seen to be effective for the decolorization of the Domalan and Bemacid families in textile effluents, especially of Green Domalan BL, as well as for achieving a significant reduction in the toxicity of the synthetic effluent after the enzymatic treatment.

4. Conclusion

The experimental results obtained in the present work reveal the effectiveness of the peroxidase-catalyzed enzymatic reaction for the treatment of textile dyes in the aqueous phase. However, the performance of the LSP-catalyzed dye-removing reaction was found to be dependent on the centrifugation and reaction times, the dye, the H_2O_2 and enzyme concentrations, and pH. Likewise, the absence of an effect of temperature on the dye equilibrium concentration was observed. The decrease in the ecotoxicity value of Green Domalan BL solutions due to the action of the LSP extract shows that the remaining products are less toxic than the starting products.

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References

1. Fu, Y. and T. Viraraghavan (2001) Fungal decolourization of dye wastewaters: A review. *Biores. Technol.* 79: 251-262.
2. Akhtar, S., A. A. Khan, and Q. Husain (2005) Potential of immobilized bitter gourd (*Momordica charantia*) peroxidases in the decolourization and removal of textile dyes from polluted wastewater and dyeing effluent.*Chemosphere* 60: 291-301.
3. Spadaro, J. T., M. H. Gold, and V. Renganathan (1992) Degradation of azo dyes by lignin-degrading fungus *Phanaerochaete chrysospotium*. *Appl. Environ. Microbiol.* 58: 2397-2401.
4. Shaffiqu, T. S., J. J. Roy, R. Aswathi, and T. E. Abraham (2002) Degradation of textile dyes mediated by plant peroxidases. *Appl. Biochem. Biotechnol.* 102-103: 315-326.
5. Gottlieb, A., C. Shaw, A. Smith, A. Wheatley, and S. Forsythe (2003) The toxicity of textile reactive azo dyes after hydrolysis and decolourisation. *J. Biotecnol.* 101: 49-56.
6. Robinson, T., G. McMullan, R. Marchant, and P. Nigam (2001) Remediation of dyes in textile effluent: A critical review on current treatment technologies with a proposed alternative. *Bioresour. Technol.* 77: 247-255.
7. Mohan, S. V. and J. Karthikeyan (1999) Removal of colour from textile dye effluents by adsorption process. pp. 272-289. In: Goel, P. K. (ed.). *Advances in Wastewater Treatment Technologies*. Technoscience Publications, Jaipur, India.
8. Karam, J. and J. A. Nicell (1997) Potential application of enzymes in waste treatment. *J. Chem. Tech. Biotechnol.* 69: 141-153.
9. Mohan, S. V., K. K. Prasad, N. C. Rao, and P. N. Sarma (2005) Acid azo dye degradation by free and immobilized horseradish peroxidase (HRP) catalyzed process. *Chemosphere* 58: 1097-1105.
10. Chivukula, M., J. T. Spadaro, and V. Renganathan (1995) Lignin peroxidase-catalyzed oxidation of sulfonated azo dyes generates novel sulfophenyl hydroperoxides. *Biochem.* 34: 7765-7772.
11. Bhunia, A., S. Durani, and P. Wangikar (2002) Horseradish peroxidase catalyzed degradation of industrially important dyes. *Biotechnol. Bioeng.* 72: 562-567.
12. Mohorcic, M., S. Teodorovic, V. Golob, and J. Fiedrich (2006) Fungal and enzymatic decolourisation of artificial textile dye baths. *Chemosphere* 63: 1709-1717.
13. Zamorano, L. S., M. G. Roig, E. Villar, and V. L. Shnyrov (2007) The versatile peroxidases. *Curr. Topics in Biochem. Res.* 9: 1-26.
14. Chen, J., S. M. Yu, and P. Zuo (2006) Horseradish peroxidase immobilized on aluminum pillared interlayered clay for the catalytic oxidation of phenolic wastewater. *Water Res.* 40: 283-290.
15. Kim, G. Y., K. B. Lee, S. H. Cho, J. Shim, and S. H. Moon (2005) Electroenzymatic degradation of azo dye using an immobilized peroxidase enzyme. *J. Hazard. Mater.* 126: 183-188.
16. Ulson de Souza, S. M. A. G., E. Forgiarini, and A. A. Ulson de Souza (2007) Toxicity of textile dyes and their degradation by the enzyme horseradish peroxidase (HRP). *J. Hazard. Mater.* 147: 1073-1078.
17. Fairbanks, G., T. L. Steck, and D. F. H. Wallach (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochem.* 10: 2606-2617.
18. Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert (1981) Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* 211: 1437-1438.
19. Bradford, M. M. (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
20. Maciel, H. P. F., C. M. C. P. Gouvêa, M. Toyama, M. Smolka, S. Marangoni, and G. M. Pastore (2007) Extraction, purification and biochemical characterization of a peroxidase from *Copaifera langsdorffii* leaves. *Quim. Nova* 30: 1067-1071.
21. Zamorano, L. S., D. G. Pina, F. Gavilanes, M. G. Roig, I. Y. Sakharov, A. P. Jadan, R. B. van Huystee, E. Villar, and V. L. Shnyrov (2004) Two-state irreversible thermal denaturation of anionic peanut (*Arachis hypogaea* L.) peroxidase. *Thermochim. Acta* 417: 67-73.
22. Wilkinson, S. R., D. J. Meyer, and J. M. Nelly (2000) Biochemical characterization of a trypanosome enzyme with glutathione-dependent peroxidase activity. *Biochem. J.* 352: 755-761.
23. Zamorano, L. S., D. G. Pina, J. B. Arellano, S. A. Bursakov, A. P. Zhadan, J. J. Calvete, L. Sanz, P. R. Nielsen, E. Villar, O. Gavel, M. G. Roig, L. Watanabe, I. Polikarpov, and V. L. Shnyrov (2008) Thermodynamic characterization of the palm tree Roystonea regia peroxidase stability. *Biochimie* 90: 1737-1749.
24. Aruldoss, J. A. and T. Viraraghavan (1998) Toxicity testing of refinery wastewater using Microtox. *Bull. Environ. Contam. Tox-*

icol. 60: 456-463.
25. Repetto, G., A. Jos, M. J. Hazen, M. L. Molero, A. Peso, M. Salguero, P. Castillo, M. C. Rodriguez-Vicente, and M. Repetto (2001) A test battery for the ecotoxicological evaluation of pentachlorophenol. *Toxicol. In vitro* 15: 503-509.
26. Johnson, T. B. (2005) Microtox acute toxicity test. *Environ.Microbiol.* 2: 69-105.
27. Hernando, M. D., S. De Vettori, M. J. Martinez Bueno, and A. R. Fernández-Alba (2007) Toxicity evaluation with *Vibrio fischeri* test of organic chemicals used in aquaculture. *Chemosphere* 68: 724-730.
28. ISO 11348-3, Water quality – Determination of inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminiscent bacteria test) – Part 3: Method using freeze-dried bacteria. 2007.
29. Onorati, F. and M. Mecozzi (2004) Effects of two diluents in the Microtox toxicity bioassay with marine sediments. *Chemosphere* 54: 679-687.
30. Klibanov, A. M., T. M. Tu, and K. P. Scott (1983) Peroxidase-catalyzed removal of phenols from coal-conversion waste waters. *Sci.* 221: 259-261.
31. Nicell, J. A., J. K. Bewtra, N. Buswas, and E. Taylor (1993) Reactor development for peroxidase catalyzed polymerization and precipitation of phenols from wastewater. *Water Res.* 27: 1629-1639.
32. Cooper, V. A. and J. A. Nicell (1996) Removal of phenols from a foundry wastewater using horseradish peroxidase. *Water Res.* 30: 954-964.
33. Veitch, N. C. (2004) Horseradish peroxidase: A modern view of a classic enzyme. *Phytochem.* 65: 249-259.
34. Roig, M. G., J. F. Bello, S. Rodríguez, J. M. Cachaza, and J. F. Kennedy (1994) Human placental alkaline phosphatase covalently immobilized on a cross-flow microfiltration polyvinylidene difluoride membrane. Part I. Physicochemical effectors. *J. Mol. Catal.* 93: 85-104.