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Short communication

Toxicity of textile dyes and their degradation by the enzyme horseradish peroxidase (HRP)

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Abstract

The enzyme peroxidase is known for its capacity to remove phenolic compounds and aromatic amines from aqueous solutions and also to decolorize textile effluents. This study evaluates the potential of the enzyme horseradish peroxidase (HRP) in the decolorization of textile dyes and effluents. Some factors such as pH and the amount of H_2O_2 and the enzyme were evaluated in order to determine the optimum conditions for the enzyme performance. For the dyes tested, the results indicated that the decolorization of the dye Remazol Turquoise Blue G 133% was approximately 59%, and 94% for the Lanaset Blue 2R; for the textile effluent, the decolorization was 52%. The tests for toxicity towards *Daphnia magna* showed that there was a reduction in toxicity after the enzymatic treatment. However, the toxicity of the textile effluent showed no change towards *Artemia salina* after the enzyme treatment. This study verifies the viability of the use of the enzyme horseradish peroxidase in the biodegradation of textile dyes.

Keywords: Textile dyes; Decolorization; Horseradish peroxidase; Toxicity; Enzyme

1. Introduction

The importance of dyes to civilization is evident and well documented. Approximately, 10,000 different dyes and pigments are in industrial use, representing an annual consumption of around 7×10^5 tonnes worldwide [1], with 26,500 in Brazil alone [2–8]. There are dyes which are recalcitrant and toxic substances, they are resistant to biological degradation, not being easily degraded in wastewater treatment plants [9].

The main techniques available in the literature for the decolorization of wastewaters involve adsorption, precipitation, chemical degradation, electrochemical, photochemical, biodegradation processes, among others [2].

Microbial decolorization has been proposed as a cheaper and less environmentally aggressive alternative. Enzymes with lignin peroxidases, manganese peroxidases and laccases have been correlated with the decolorization of dyes [6]. Enzymes can act on specific recalcitrant pollutants to be removed by precipitation or transformation them into other innocuous products [2,3,8–15].

In the biological removal of color from effluents, the employment of fungi and of their oxidative enzymes constitutes an alternative for treatment under aerobic conditions [16]. The oxidative degradation of colored compounds is significantly stimulated through the use of oxidative enzymes [2–5,8–19].

The enzyme horseradish peroxidase (donor: hydrogen peroxide oxido-reductase (EC: 1.11.1.7, HRP)) is a versatile enzyme applied in the chemical, environmental, pharmaceutical and biotechnological industries [12]. Horseradish peroxidase (HRP) is known to be effective in the removal of a wide spectrum of aromatic compounds (phenols, biphenols, anilines) in the presence of H_2O_2 [18] and in the degradation and precipitation of important industrial azo dyes [11,14].

Many treatments can be efficient in decolorization, however, it is necessary to evaluate whether there is the formation of toxic products during the treatment process. One valuable technique

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to evaluate the efficiency of a degradation process is the use of bioindicators [19]. In this study, the use of the enzyme HRP in the decolorization of textile dyes and effluents is discussed. Some parameters were studied to improve the degradation efficiency, and the toxicity of the raw textile effluent and the effluent after post-enzymatic treatment with the use of the bioindicators *Artemia salina* and *Daphnia magna*, were evaluated.

2. Experimental

Initially, the capacity of the enzyme peroxidase to remove color from dyes was studied and later the performance of the enzyme in the decolorization of textile effluents and the toxicity of the effluent were determined.

2.1. Peroxidase enzyme

The enzyme horseradish peroxidase, used in this study, was produced by Toyobo do Brasil. The enzymatic solution was prepared in citrate–phosphate buffer (pH 5.0 and 50 mmol L^{-1}); before each experiment it was centrifuged and the supernatant was used, and stored at 5 °C.

2.2. Dyes

The dyes studied were Remazol Turquoise Blue G 133% and Lanaset Blue 2R. Each dye was diluted in distilled water, up to a concentration of 100 mg L^{-1}, giving a stock solution from which aliquots were taken for the decolorization experiments. The dyes were used without prior purification.

2.3. Textile effluent

The textile effluent studied was collected from a textile industry, located in the State of Santa Catarina. The sample was kept at an ambient temperature, the original pH being 4.0. The effluent was collected at the outlet of the neutralization tank, and was used without any prior treatment.

2.4. Enzyme assay

The determination of the activity of the enzyme horseradish peroxidase, was carried out using the method of Szklarz et al. [20] with modifications, employing syringaldazine (Sigma) as the enzymatic substrate. The reaction mixture was composed of 0.6 mL of enzymatic solution, 0.2 mL of citrate–phosphate buffer (50 mmol L^{-1}, pH 5.0), 0.1 mL of H_2O_2 (2.0 mmol L^{-1}) and 0.1 mL of syringaldazine (0.1% in ethanol). The oxidation of syringaldazine was accompanied by spectrophotometric analysis, at zero time and after 10 min, at 525 nm ($\varepsilon_{525\,nm}$ 65000 L/M cm).

One unit of enzyme activity was defined as the amount of enzyme which oxidized 1 μmol of substrate per minute. The activity of the free purified enzyme was 29.85 U mL^{-1}.

2.5. Enzymatic treatment

To 100 mL of aqueous solution of the dyes or textile effluent, 5×10^{-1} mL of enzymatic solution were added, with a concentration of 29.85 U mL^{-1}, corresponding to 0.1485 U mL^{-1} of solution, and 2×10^{-3} mmol L^{-1} of H_2O_2. The enzymatic reaction was carried out under shaking in a horizontal shaker (Nova Ética) at 100 ppm. The experiments were conducted at a constant temperature of 25 °C. Aliquots were removed every 10 min, being centrifuged and submitted to analytical control.

2.6. Analytical procedures

The evaluations of the decolorization efficiencies were carried out through absorbance readings at the maximum wavelengths (λ_{max}) of 624 nm (Turquoise) and 590 nm (Blue 2R), using a Shimadzu UV mini-1240 spectrophotometer. The quantification of the textile effluent color was carried out using the area below the curve, obtained using a spectral scanner in the range 200–800 nm, and the decolorization was determined through the difference between the initial and final areas.

The pH measurements were carried out by potentiometry, using a Digimed pH meter, calibrated with standard solutions of pH 4.0 and 7.0, and the method recommended by the Standard Methods for Examination of Water and Wastewater [21].

2.7. Acute toxicity test with A. salina

The acute toxicity tests were carried out according to the Matthews method [13] with modifications. The methodology of the toxicity test with *A. salina* consisted of exposing the nauplii with 24 h of life, after being kept in saline solution (32 g L^{-1}), to different concentrations of the textile effluent before and after enzymatic treatment, for a period of 24 h at 25 °C. The effluents were tested in a concentration series (100, 75, 50, 25, and 10%) in order to obtain the lethal concentration 50 (LC_{50}). Negative controls were carried out in parallel using only a synthetic marine salt solution. After 24 h of incubation, a number count of the dead larvae was carried out and the LC_{50} was calculated. The tests were carried out in quadruplicate.

2.8. Acute toxicity test with D. magna

The acute toxicity tests with *D. magna* were carried out according to the ABNT norms [22]. 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 a duration of 48 h, and after 24 h 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.

Table 1
Study for the determination of the optimum conditions for the performance of the enzyme HRP

Parameters	Range evaluated	Optimized parameters
pH	2.0–9.0	4.0–5.0
Dye concentration (mg L^{-1})	10–100	100
Quantity of enzyme (U mL^{-1})	2.985–29.85	29.85
Quantity of H_2O_2 (mmol L^{-1})	1×10^{-3} to 1.2×10^{-2}	2×10^{-3}

3. Results and discussion

3.1. Results for the dye degradation with the enzyme HRP

The objective of this study is to obtain the maximum degradation percentage with the minimum quantity of inputs, minimizing the process costs. In this paper, the parameters were optimized separately (pH, dye concentration, quantity of H_2O_2, quantity of enzyme, and reaction time), possible interactions between the parameters not being considered. Further studies will be carried out to determine the interaction between these parameters and the degradation process efficiency.

In Table 1, the data evaluated in the optimization study carried out with the dye Remazol Turquoise Blue G 133% are shown, in order to obtain the optimum conditions for the enzyme performance in the decolorization of the textile dye and effluent.

Most enzymes have a characteristic pH value at which their activity is maximized. The interrelation of enzymatic activity with pH, for any enzyme, depends on the acidic–basic behavior of the substrate, as well as other factors which are, in general, difficult to analyze quantitatively. The enzyme HRP showed a better performance at pH 4.0, obtaining 59% of decolorization, and with an increase in pH the decolorization efficiency was reduced (Table 1). Bhunia et al. [17] studied the enzymatic decolorization of the dyes Remazol Blue and Red Cibacron, at different pH values, and it was concluded that at pH values above 6.0, the HRP activity was inhibited. Tong et al. [23] obtained the maximum HRP activity in the pH range of 5–9, the optimum pH being 7.0. From these results it can be concluded that the ideal pH may vary for the same enzyme, highlighting the need to study the pH to be used.

In the optimization of H_2O_2 quantity, evaluations were carried out in the absence of H_2O_2 and using concentrations of 1×10^{-3} to 1.2×10^{-2} mmol L^{-1}. It was observed that in the absence of this coadjutant there was no decolorization, and the concentration which showed a better enzyme performance 2×10^{-3} mmol L^{-1} (59% of decolorization), began to have an inhibitory effect.

Normally, the removal of an aromatic compound is dependent on the concentration of the enzyme added, considering that the catalyst and also the conversion are dependent on the contact time and the respective optimum performance parameters (pH, temperature, and substrate). An optimum relation between the quantity of enzyme and the substrate may achieve a maximum activity. Within the enzyme concentrations evaluated (2.985–29.85 U mL^{-1}), it was observed that when the concentration studied was 14.985 U mL^{-1}, the decolorization of the dye was 58%; however, when the concentration was doubled, the decolorization was 62%. From these results it was concluded that often, using a higher concentration of enzyme, the decolorization of the dye was not significantly influenced, for the experimental conditions specified.

The optimization of the quantity of enzyme was carried out aiming at a high efficiency of decolorization with a lower enzyme quantity used in the process in the free form. Under the experimental conditions studied, the enzyme deactivation was not observed, since the enzyme activity remained practically the same, before and after the experiment.

The quantity of peroxide and of enzyme, which were optimized in this study, did not influence the results, when other parameters were evaluated, and also when working with the textile effluent. However, if other types of dyes and the enzyme in the immobilized form are used, it will be necessary to carry out a new optimization study.

Another important factor is the catalyst/substrate contact time which gives a maximum removal. According to Lui et al. [9], 5 min are sufficient to obtain a degradation of the dyes bromophenol blue and methyl orange, with decolorization of 100 and 80%, respectively. However, Mohan et al. [14] have reported that 45 min is the reaction time required to catalyze the degra-

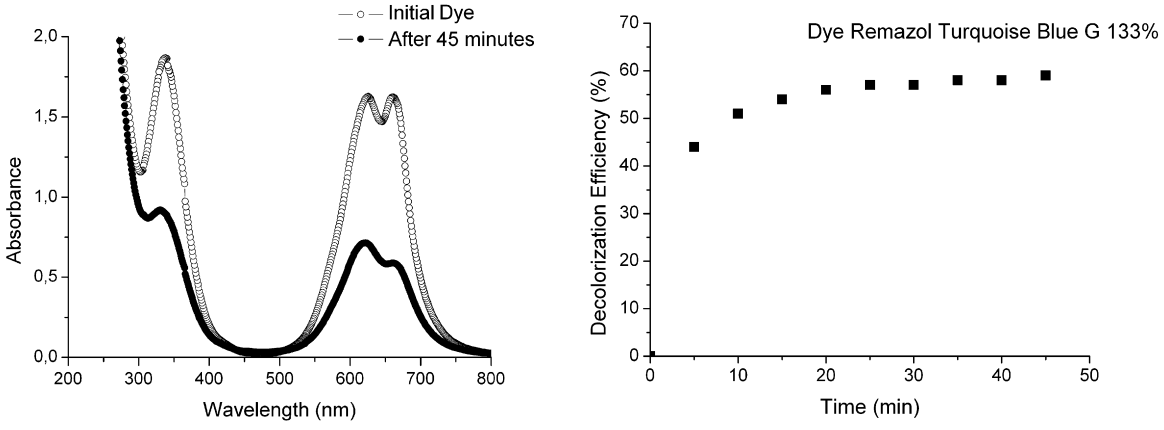

Fig. 1. Scanning spectrum of the dye Remazol Turquoise Blue G 133% and the effect of reaction time on the decolorization of the dye.

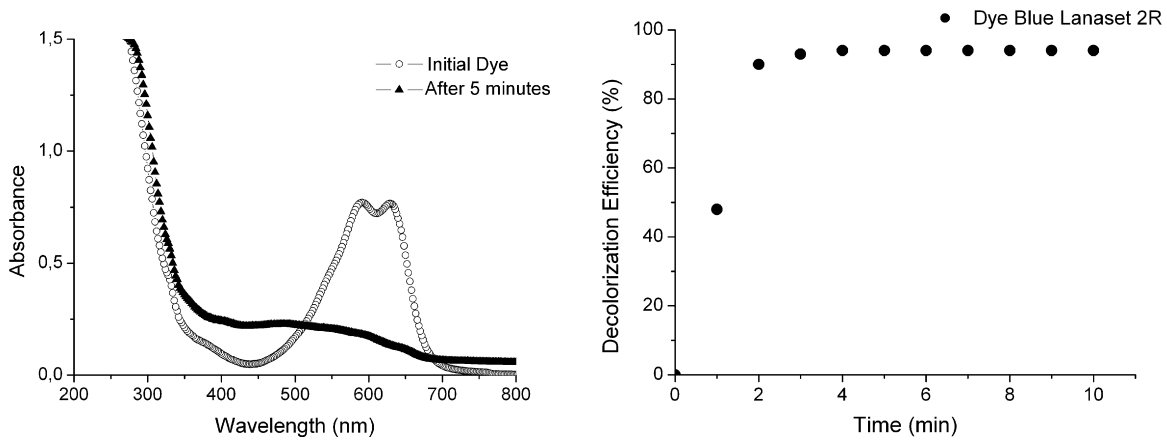

Fig. 2. Scanning spectrum of the dye Blue Lanaset 2R before and after enzymatic treatment. Effect of reaction time on the decolorization of the dye.

dation of the dye Acid Black 10 BX, and that after 5 min, the removal reaction followed a slower process, and the decolorization was 67% with HRP in the free form. The fact that the reaction became slower may be attributed to the simultaneous decrease of all species which are reacting (dye, HRP and H_2O_2).

The efficiencies of the decolorization of the dyes Turquoise Blue G 133% and Lanaset Blue 2R, as a function of contact time with the enzyme, are given in Figs. 1 and 2. Different reaction times were observed for the dye Lanaset Blue 2R, and in 5 min of contact with the enzyme the decolorization was 94%, and 59% for Turquoise Blue G 133%, after 45 min of contact with the enzyme (Fig. 1). We can conclude that the enzymatic reaction of HRP is fairly rapid, with most of the color removed in the first 5 min of contact with the enzyme. In similar studies with the enzyme horseradish peroxidase, Bhunia et al. [17] evaluated that most of the degradation of the dye Remazol Blue, occurred within 3 h.

Since the commercial dyes have a variety of colors and structures, the enzyme will act in a different way, both in relation to its removal capacity and the degradation rate.

3.2. Results for the textile effluent decolorization with the enzyme HRP

Textile wastewaters are characterized as having a strong color, since some of the initial dye (10–15%) is not fixed to the fiber during the dyeing process, thus being released to the effluents. When released to the environment without treatment, they can cause serious contamination problems, decreasing the water transparency and, consequently, inhibiting the penetration of solar radiation and decreasing photosynthesis.

In Fig. 3, the scanning spectra of the textile effluent before and after enzymatic treatment are shown. The tests were carried out at pH 4.0, 2×10^{-3} mmol L^{-1} of H_2O_2, at 25 °C and under shaking at 100 rpm. Every 10 min a sample was collected, centrifuged and the spectrophotometric reading taken. The experiments were carried out following a procedure similar to that used for the pure dyes, due to the Remazol Turquoise G 133% dye being present in the effluent. However, the characteristics of the textile effluent are different due to the presence of auxilliary chemical substances used in the process.

The quantification of the decolorization efficiency was carried out through the difference in the area under the curve, and a decolorization of 52% was obtained.

The formation of a new peak can be observed in the region between the wavelengths 350 and 450 nm. This new peak is probably due to the formation of intermediate compounds during the enzymatic treatment, resulting from the incomplete degradation of the effluent. On quantifying the efficiency of the effluent decoloration, this new peak was also considered, since the percentage of effluent decoloration was calculated as a function of the area below the curve of the spectrum. The enzyme HRP showed a good color removal from the textile effluent.

With the aim of evaluating the degradability of the effluent by the enzyme, COD tests were carried out before and after enzymatic treatment. The COD values for the two effluents were similar, thus indicating that the enzymatic process acts only on the chromophore group, breaking the dye molecule. The enzymatic process may be used as a pretreatment, requiring a post treatment for the removal of the organic load present in the effluent.

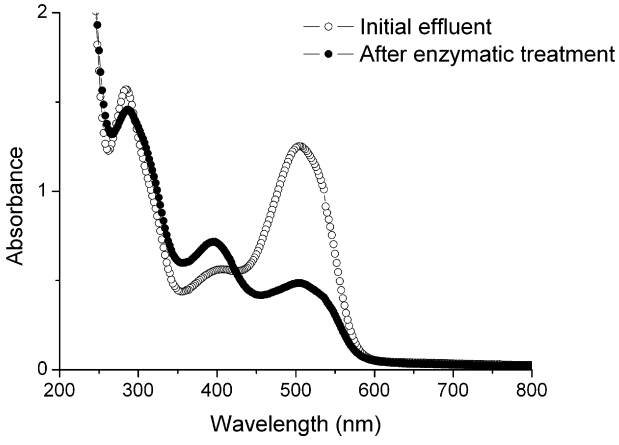

Fig. 3. Scanning spectra of textile effluent, before and after treatment with the enzyme HRP in the free form.

Table 2
Acute toxicity tests with the microcrustacean *Artemia salina*, after 24 h of incubation in different effluent concentrations

Effluent concentration (%)	Non-treated effluent mortality (%)	Treated effluent mortality (%)
100	100 ± 0	100 ± 0
75	90 ± 0.7071	90 ± 0.5773
50	40 ± 0.4082	30 ± 1.0
25	0 ± 0	0 ± 0
10	0 ± 0	0 ± 0

*LC_{50} = 55% (non-treated effluent); *LC_{50} = 58.33% (treated effluent).

In this study, results were obtained with the HRP enzyme in the free form. In order to analyze the possibility for the scale-up of the laboratory process to a larger scale, new studies need to be carried out on the efficiency of the enzymatic degradation process, using the HRP enzyme in the immobilized form.

3.3. Toxicity tests

The response established for the acute toxicity test with *A. salina* was the average lethal concentration (LC_{50}) at which 50% of the larvae were killed during 24 h of exposure to the effluent. The criteria used for the acute toxicity test with *D. magna* was the dilution factor (DF), which establishes the effluent concentration that can cause the mortality or immobility of over 10% of the exposed organisms during a 48-h period at different effluent concentrations.

The toxicity study of the raw textile effluent and the effluent after the enzymatic treatment was carried out with the purpose of evaluating whether the reaction products would be more toxic than the raw effluent.

From the results for *A. salina* shown in Table 2, the toxicity of the effluent before and after enzymatic decolorization treatment, it can be observed that the non-treated effluent showed an average lethal concentration (LC_{50}) of 55%, and after treatment it was 58.33%. The effluent concentration which showed the lowest mortality was 50%, with 40% of *A. salina* larvae being dead before the enzymatic treatment and 30% of larvae after. The results showed that there was no significant change in the acute toxicity in relation to the test organism.

Table 3
Results for *Daphnia magna* exposed to the raw and after enzymatic treatment, after 48 h of incubation

Dilution	Effluent concentration (%)	Mortality (%) raw textile effluent	Mortality (%) after enzymatic treatment
1	100	100 ± 0	100 ± 0
2	50	100 ± 0	100 ± 0
3	33.3	100 ± 0	40 ± 11.51
4	25	100	15 ± 9.57
6	16.6	60 ± 26.29	5 ± 5.77
12	8.3	50 ± 0	0 ± 0
24	4.2	10 ± 11.57	0 ± 0
48	2.08	0 ± 0	0 ± 0

The tests were carried out in duplicate, $n = 10$. The effluent dilution was carried out from a concentration of 100%.

According to Table 3, which presents the results for *D. magna*, it can be observed that for the treated effluent, the toxicity factor was below 6 (DF), since for this dilution the average mortality obtained was 5%, and for the raw effluent it was for a dilution of 24, which gave the lowest mortality (10%). From these results it can be concluded that the toxicity factor of the effluent after enzymatic treatment is at least four times lower than the toxicity factor of the non-treated effluent. Thus, the textile effluent remediation with the enzyme HRP managed to reduce significantly the toxicity of the effluent under study, decreasing greatly the mortality of the daphnia exposed to the treated samples.

The acute toxicity tests for the textile effluent with the bioindicator *D. magna* showed that there was a reduction in the toxicity of the textile effluent after the treatment with the horseradish peroxidase enzyme. However, the toxicity of the textile effluent showed no change towards *A. salina* after the enzyme treatment. Each bioindicator used responded differently when in contact with the effluent under study, which is related to the sensitivity of the organism to the toxic compounds.

The enzyme HRP, in the free form, was shown to be effective for the decolorization of textile dyes and effluents, as well as for achieving a reduction in the toxicity of the effluent after the enzyme treatment.

4. Conclusions

The enzyme horseradish peroxidase showed a good decolorization of textile dyes. The acute toxicity tests for the textile effluent with *D. magna* showed that there was a toxicity reduction of the textile effluent after treatment with HRP. However, the toxicity of the textile effluent showed no change towards *A. salina* after the enzyme treatment. The two bioindicators used responded in a different way when in contact with the effluent under study, however, this is related to the sensitivity of the organism to the toxic compounds. It is here concluded that the effluent is toxic to various organisms, however, it can be detoxified through degradation by the enzyme HRP.

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