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Decolorization of dyes with different molecular properties using free and immobilized laccases from *Trametes versicolor*

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ABSTRACT

Laccases from *Trametes versicolor* have been used for the decolorization of acid and reactive dye solutions. The dyes considered were the acid orange 7 (AO7), acid blue 74 (AB74), reactive red 2 (RR2) and reactive black 5 (RB5). The decolorization of dye solutions was performed in batch systems under constant agitation at 30 °C, using both free laccases and laccases immobilized on a mesoporous carbon obtained from pecan shells. Results obtained pointed out that the biocatalysts tested are more effective in the decolorization of acid dyes (AO7 and AB74), with more than 90% of decolorization percentages attained, than reactive dyes. Furthermore, the laccase used in this study, either freely dissolved or supported on the carbon materials, was inactive towards RB5, which was the larger dye molecule (992 g mol^{-1}). Immobilization of the laccase on the mesoporous carbons brought about a considerable decrease of their activity. However, an enzyme stabilization effect was also observed as the activity of the supported laccase was maintained over a wide pH span. Finally, the combination of spectrophotometric and chromatographic (HPLC) techniques allowed us to get a more precise picture of the influence of adsorption/oxidation mechanisms during the degradation of dyes by the supported enzymes.

1. Introduction

Currently, the development of efficient treatment and bioremediation technologies for the control and reduction of water pollution is one of the most important challenges for researchers. The discharge of wastewater from textile, paper, leather tanning, food processing, plastics, cosmetics, rubber, printing and dye manufacturing industries into water resources is a significant cause of water pollution due to the presence of synthetic dyes [1]. These dyes represent an environmental concern for their high visibility, recalcitrance and toxic impact [2]. The presence of dyes in water can affect the aquatic plants because they reduce sunlight transmission through water. Dyes are also responsible of direct and indirect toxic effects on humans. Specifically, the exposition of humans to dyes has been linked to jaundice and tumor development, skin irritation, allergies, heart defects and mutations [3,4].

Nowadays, there are several physico-chemical techniques for the treatment of wastewaters from textile industry, including: adsorption, coagulation, flocculation, precipitation, ion exchange, froth flotation, membrane separation and electrochemical methods [5–7]. However, the resistance of many toxic dyes to these techniques has led researchers to study alternative methods for the degradation of hazardous dyes [6]. The use of enzymatic biodegradation has demonstrated to be potentially effective in the treatment of this pollution source in an eco-efficient manner [8]. Furthermore, the immobilization of enzymes on different supports allows the biocatalysts to be reused many times [9]. Oxidative enzymes from various sources (fungi, bacteria, plants) have been studied and the results indicate that these enzymes perform outstandingly in treating wastes from pulp and paper industry [10]. Particularly, laccases (EC 1.10.3.2, benzenediol: oxygen oxidoreductases), have shown a great potential in this area. Laccases have four copper atoms, one paramagnetic type 1 copper (T1 Cu) that is responsible for their characteristic blue color and where the oxidation of the reducing substrate occurs. On the other hand, the one type 2 copper (T2 Cu) and two type 3 coppers (T3 Cu) conform a trinuclear cluster in which molecular oxygen is reduced to two molecules of water [11].

Laccases catalyze the oxidation of a wide number of phenolic compounds and aromatic amines but their substrate range have been extended to non-phenolic compounds in the presence of low molecular mass compounds acting as mediators [12]. The major drawback of the enzymatic systems used in different applications is their low stability and productivity, as well as their high production costs [13]. However,

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enzyme immobilization improves the economy of biocatalytic processes by enzyme reuse and the eventual increase of enzyme stability [14]. Laccase has been successfully immobilized on different supports, including activated carbon, alginate-chitosan microcapsules, hydrogel structures and mesoporous silica [15–18]. Enzyme immobilization by adsorption is a popular strategy for most large-scale applications due to ease in catalyst recycling, continuous operation, product purification, and the stability of the enzyme activity after adsorption [19]. Also, the biocatalytic efficiency can be improved by manipulating the structure and/or chemistry of the carriers, being mesoporous materials excellent candidates for enzyme immobilization due to the good connectivity of the porous network, which offers the possibility of minimizing the diffusional hindrance during the catalytic performance that usually results in a loss of activity of the immobilized enzyme [20]. Besides, the use of activated carbon for this purpose becomes attractive for its versatility, large surface area, polymodal porosity, high adsorption capacity and variable surface chemical composition [2].

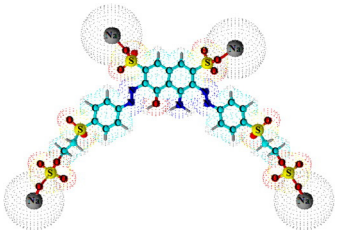
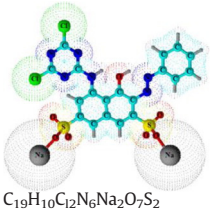
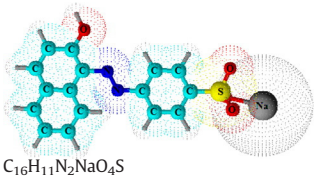
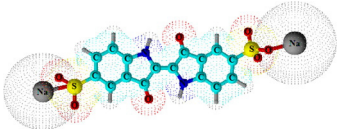
This work aims to study the decolorization of dyes with different chemical structures using laccases from *Trametes versicolor*. Tests were carried out using both free enzyme and laccase supported on mesoporous carbons obtained from pecan nut (*Carya illinoinensis*) shells.

2. Materials and methods

2.1. Dyes selected

The dyes studied in this work were the acid orange 7 (AO7), acid blue 74 (AB74), reactive red 2 (RR2) and reactive black 5 (RB5). All dyes were purchased from Aldrich Company. Selected properties of these dyes are shown in Table 1. All dyes were used as received, *i.e.*, without further purification.

Table 1
General characteristics of the studied dyes.

Dye and code assigned	Structure	MW (g mol^{-1})	λ_{max} (nm)
Reactive black 5 (RB5)	 $C_{26}H_{21}N_5Na_4O_{19}S_6$	991.82	597
Reactive red 2 (RR2)	 $C_{19}H_{10}C_{l2}N_6Na_2O_7S_2$	615.33	538
Acid orange 7 (AO7)	 $C_{16}H_{11}N_2NaO_4S$	350.32	484
Acid blue 74 (AB74)	 $C_{16}H_8N_2Na_2O_8S_2$	466.35	610

2.2. Free and supported enzyme activity studies

Laccase from *T. versicolor* (specific activity ≥ 10 U mg^{-1}) was provided from Sigma-Aldrich Company (CAS Number 80498-15-3), stored at −20 °C and used as-received. One unit of enzyme activity (U) is defined as the amount of enzyme that oxidizes 1 μmol of ABTS to colored products (420 nm, ε = 36,000 M^{-1} cm^{-1}) per minute.

The support used for the immobilization process was a mesoporous carbon obtained from pecan nut (*C. illinoinensis*) shells. This carbon was prepared as reported elsewhere [21]. The immobilization of laccase from *T. versicolor* was carried out by adsorption in batch systems under constant agitation (150 rpm) at 25 °C. The laccase was dissolved in a phosphate buffer (100 mM; pH 6) to obtain an initial concentration of 3 mg ml^{-1}. The solution (5 ml) was mixed with 50 mg of carbon support and after 48 h, the concentration of enzyme in the resulting suspension was analyzed. The initial and final concentration of enzyme was determined according with the Bio-Rad protein assay, based on the Bradford method [22], which makes use of the bovine serum albumin (BSA) as standard protein for the calibration curve. This method involves the binding of Coomassie Brilliant Blue G-250 to protein, which causes a shift in the maximum absorption wavelength of the dye from 465 to 595 nm. The light absorption at 595 nm was monitored using a Hach DR-5000 spectrophotometer. The immobilization percentage was calculated by measuring the protein concentration in the initial and final laccase solution as follows:

$$\%\,\text{immobilization} = \left(1-\left(\frac{\text{Final protein concentration}}{\text{Initial protein concentration}}\right)\right)\times 100 \quad (1)$$

To test the strength of the enzyme/support interaction, four cycles of desorption were carried out. The enzyme/support systems were thus suspended on 5 ml of phosphate buffer at pH 6, under constant shaking (150 rpm) for 24 h at 25 °C, for each cycle. The amount of protein desorbed was then analyzed as described above. After four desorption cycles, the carbon with enzyme was placed in the oven at 25 °C during 24 h.

The catalytic activity at different pH of free and immobilized laccase was determined spectrophotometrically (Hach DR-5000 spectrophotometer) from the initial reaction rate region with ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) as substrate [23]. The reaction mixture for free enzyme consisted of 3.8 ml of 2 mM ABTS and 0.2 ml of laccase solution dissolved in 50 mM acetate buffer (pH 3–5) and 50 mM phosphate buffer (pH 6–8). The enzyme solution was added to the cuvette containing the substrate solution, which was placed into the spectrophotometer after a swift agitation. The reaction was monitored continuously for 1 min by measuring the absorption at 420 nm, at room temperature.

To evaluate the enzymatic activity of the immobilized laccase, 20 mg of biocatalyst were suspended in 5 ml of buffer. Then, 5 ml of ABTS (2 mM) were added under stirring and aliquots were taken from the reactor at 30 s intervals at room temperature in batch regime. After the absorbance measurements, the aliquots were returned to the reactor.

2.3. Dye decolorization studies

Two types of decolorization tests were performed in this study; the first type was performed using the free enzyme and the second type with the enzyme immobilized on a mesoporous carbon. The experimental details are described below.

2.3.1. Decolorization studies using free laccases

The decolorization tests were performed in batch reactors at 25 °C under constant agitation (150 rpm) using an incubator (Lab Companion