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Immobilization of commercial laccase onto green coconut fiber by adsorption and its application for reactive textile dyes degradation

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ABSTRACT

An effort has been made to find a cheaper, easily available and simple alternative for the immobilization of enzymes and subsequent utilization at large scale in textile wastewater treatment. Commercial laccase was immobilized for the first time on an agroindustrial residue, green coconut fiber, by physical adsorption. The effect of the immobilization conditions (enzyme concentration, contact time and pH value) on the properties of the biocatalyst was determined. Then, the immobilized enzyme characterization was performed and kinetic parameters were obtained. Thermal and operational stabilities were improved compared with free commercial laccase showing its potential for continuous applications. Finally, the performance of immobilized laccase for the continuous degradation of various reactive textile dyes and of a mixture of them in batch reactors was evaluated. Two phenomena were observed: decolourization of the solutions due to dyes adsorption on the support and due to the enzyme action. A high decolourization percentage of practically all dyes in the first two cycles and an effective decolourization of the dye mixture were obtained, showing the suitability of the immobilized commercial laccase for continuous colour removal from textile industrial effluents.

1. Introduction

A large number of synthetic dyes are being increasingly used in the textile, paper, pharmaceutical, cosmetics and food industries. Over 7×10^5 t of approximately 100,000 different dyes and pigments are produced annually worldwide, of which about 50,000 t are discharged into the environment [1]. These compounds cause serious environmental pollution. Most of them are toxic, mutagenic and carcinogenic. Moreover, they are unusually resistant to degradation due to their complex structure and synthetic origin. Colour can be removed from effluents by chemical and physical methods including adsorption, coagulation–flocculation, ion-exchange, oxidation and electrochemical methods [2]. However these methods have financial and methodological disadvantages. They are also time-consuming and are mostly ineffective. Alternatively, dye decolourization using microbial enzymes, such as laccases, has received great attention in recent years due to its efficient application [3–6]. Laccase-based decolourization treatments are potentially advantageous to bioremediation technologies since the enzyme is produced in larger amounts for industrial applications [7]. Laccase (p-diphenol oxidase, EC 1.10.3.2) catalyzes the oxidation of phenolic compounds and aromatic amines and accepts a broad range of substrates [8]. The number of substrates can further be extended by using laccase in combination with mediators [9].

One of the main drawbacks of using free enzymes to detoxify waste streams is their instability towards thermal and pH denaturation, non-reusability, proteolysis, and inactivation by inhibitors. The immobilization of enzymes to water-insoluble supports can increase their operational stability and durability and can provide easy recovery of the enzyme. Furthermore, enzyme immobilization would allow the reuse of the enzyme and thus decrease the cost of industrial applications and allow process control. Therefore, many efforts have been made to immobilize laccase from various sources [10,11], but most of the immobilized enzyme preparations either use commercially available enzyme or expensive supports, which increase the cost of the processes [12].

Several techniques may be applied to immobilize enzymes on solid supports. They are mainly based on chemical and physical mechanisms [11]. Therefore, it is hardly surprising that there is no general universally applicable method of enzyme immobilization. The main task is to select a suitable carrier, condition (pH, temperature and nature of medium) and the enzyme itself (source,

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nature and purity) to design an immobilized biocatalyst. The selected method should meet both the catalytic needs (expressed in productivity, space–time yield, stability and selectivity) and the non-catalytic needs (e.g., separation, control, down-streaming process) that are required by a given application. As a result, an immobilized enzyme can be labeled as "robust", when both the catalytic and the non-catalytic functions can meet the requirements of a specific application [13].

Laccase, immobilized on several supports, has been evaluated for the elimination of pollutants such as phenols, but few studies have investigated dye decolourization [11]. Dye decolourization using laccase immobilized on imidazol-modified silica gel [14] or silanized alumina particles [15] occurred mainly by adsorption and to a lesser extent, by enzymatic decolourization. More recently, Champagne and Ramsay [16] demonstrated that laccase immobilized on controlled porosity carrier glass beads using APTES-glutaraldehyde decolourized a single anthraquinone dye mainly by enzymatic degradation.

An effort has been made to find a cheaper and easily available alternative for the immobilization and subsequent utilization at large scale of commercially available enzymes. Thus, green coconut fiber was herein used as support for laccase immobilization. To our knowledge, no reports on laccase immobilization by physical adsorption on green coconut fiber have been previously published. Among the various immobilization techniques, the physical adsorption on the basis of bioaffinity may be a good choice for enzyme immobilization, as this process can immobilize enzyme directly from crude homogenate and thus avoid the high cost of purification. It saves time and labor since it is a simple operation, no further treatment of the support is needed and supports can be reused after desorption of the inactivated enzyme, thus reducing the final price and generating fewer residues [17]. The ease of immobilization, lack of chemical modification and enhancement in stability are some of the advantages offered by the adsorption procedures [12]. Besides the mentioned advantages offered by the bioaffinity-based procedures, there is an additional benefit, such as proper orientation of enzyme on the support [18]. These supports provide high yield and stable immobilization of enzymes. However, this technique possesses disadvantages, such as low linking energy between enzyme and support, which may cause enzyme desorption in presence of the substrate or when it is exposed to variations on temperature, pH and ionic strength. Nevertheless, immobilization by adsorption is one of the most used techniques in the attainment of insoluble biocatalysts [19].

The first objective of this study was to immobilize the commercial laccase by adsorption on green coconut fiber and to determine the effect of the immobilization conditions (enzyme concentration, contact time and pH value) on the properties of the biocatalyst. The second objective was to evaluate the performance of immobilized laccase regarding the continuous degradation of various reactive textile dyes and of a mixture of them in batch reactors.

2. Materials and methods

2.1. Chemicals and enzyme

2.1.1. Textile dyes

Reactive Black 5 (RB5) (Remazol Black B), Reactive Blue 114 (RB114) (Levafix Brilliant Blue E-BRA), Reactive Yellow 15 (RY15) (Remazol Yellow GR), Reactive Yellow 176 (RY176) (Remazol Yellow 3RS), Reactive Red 239 (RR239) (Remazol Brilliant Red 3BS) and Reactive Red 180 (RR180) (Remazol Brilliant Red F3B) were kindly provided by DyStar (Portugal) and were used for degradation experiments without any further purification.

2.1.2. Enzyme

Commercial laccase formulation (DeniLite IIS; 120 U/g) from genetically modified *Aspergillus* was kindly provided by Novozymes. This formulation is used for indigo dye decolourization in denim finishing operations and includes a buffer and an enzyme mediator, but this information was not indicated by Novozymes.

2.1.3. Support

Green coconut fiber was kindly donated by Embrapa Agroindústria Tropical, Ceará State, Brazil. It was cut and sieved to obtain particles between 32 and 35 mesh and then washed with abundant distilled water and dried at 60 °C during 24 h before being used as immobilization matrix.

2.2. Immobilization procedures

To optimize the enzyme immobilization conditions by adsorption technique, 0.15 g of green coconut fiber were incubated in syringes of 5.0 mL with 1.5 mL of laccase solution containing 0.008–2.6 g/mL of enzyme at room temperature and varying pH from 4.0 to 9.0 at room temperature. The syringes with the solutions were stirred on a rotational shaker TECNAL, model TE-165. At the optimized conditions of pH and enzyme concentration, the effect of contact time was evaluated in the range 30 min–8 h. The solutions were also stirred on the rotational shaker. After immobilization, the support was separated by filtration and washed three times with 0.1 M of phosphate buffer (pH 7.0) (±100 mL each wash). The supernatant was kept for enzyme activity measurements. The immobilized enzyme activity was measured as described below. For each assay duplicate or triplicate runs were made.

2.3. Measurements of activity of free and immobilized laccase

The free laccase activity was assayed spectrophotometrically (Thermo Electron, model UV1 spectrophotometer) with ABTS as substrate (0.4 mM) in 0.05 mM citrate/0.1 mM phosphate buffer at pH 4.5. To measure the laccase activity, 0.1 mL of the incubated enzyme solution was added to 1.9 mL of the ABTS solution at 40 °C [20]. The change in absorbance at 420 nm ($\varepsilon = 36 \times 10^3\ M^{-1}\ cm^{-1}$) was recorded for 30 s and the catalytic activity was determined by measuring the slope of the initial linear portion of the kinetic curve. One unit (U) was defined as the amount of enzyme that oxidized 1 μmol of ABTS per min. and the activities were expressed in U/L.

The immobilized laccase activity was assayed by incubating 0.1 g of support in 7.0 mL of the same citrate–phosphate buffer with 2.5 mL of ABTS (0.4 mM) at 40 °C. The change in absorbance was monitored every minute, over a 4 min period, by withdrawing 2 mL of the solution. After rapid measurement without temperature control in the spectrophotometer, the solution was turned back to the reactor. The absorbance at 420 nm ($\varepsilon = 36 \times 10^3\ M^{-1}\ cm^{-1}$) was measured as before. The final activity of immobilized laccase was expressed in U/kg.

Immobilization yield (%) is defined as the difference between enzyme activity in the supernatant before and after immobilization divided by the enzyme activity in the supernatant before immobilization.

The recovered activity (%) of the immobilized enzyme is defined as the ratio between the activity of the immobilized enzyme and the activity of a similar amount of the free enzyme.

2.4. Thermal stability of free and immobilized laccase

The thermal inactivation of the free and immobilized laccase was investigated by incubating the free and immobilized enzyme in phosphate (100 mM) buffer pH 7.0 at 60 °C. For this purpose 0.1 g of immobilized enzyme or free laccase (550 U/L) were incubated in a water bath with temperature control. In certain time intervals consecutive aliquots were taken up to complete inactivation. The initial activities were compared with the residual activities. The thermal parameters were calculated according to the simplified deactivation model proposed by Henley and Sadana (1985) referenced by Arroyo et al. [21]:

$$A = (1 - \alpha)e^{-kt} + \alpha \quad (1)$$

where A is the residual enzyme activity, α is the ratio of specific activity E_1/E to the different states (see Eq. (2)), k is the thermal inactivation parameter and t is the time. Thermal parameters were estimated by fitting the experimental data to Eq. (1) using a non-linear regression code MicroCal Origin software v.3.01 (SPSS Inc., Northampton, USA).

$$E \xrightarrow{k} E_1 \quad (2)$$

Biocatalyst half-life ($t_{1/2}$) was calculated from Eq. (1), using the estimated parameters (k and α) and making A equal to 0.5. In this work, stabilization factor (F) was considered as the ratio of immobilized enzymeĭs half-lives to soluble enzyme half-life.

2.5. Operational stability of immobilized laccase

Operational stability of the immobilized laccase was assessed by incubating 0.1 g of the immobilized laccase with 2.5 mL of ABTS (0.4 mM) in 7 mL of citrate–phosphate buffer pH 4.5 at room temperature and under moderate mixing. 16 cycles of operational stability were carried out. At each cycle, a sample was withdrawn in 1-min intervals, absorbance was measured and then it was returned to reactor (initial reaction rate measurements). Afterwards, the reaction was stopped to the substrate removal. Then the immobilized enzyme was collected by filtration, washed twice with phosphate buffer 100 mM pH 7.0 (±100 mL each wash) and resuspended in a fresh substrate solution to begin the next cycle. For each assay duplicate runs were made.

2.6. Determination of kinetic parameters

The Michaelis–Menten kinetic parameters K_M and v_{max} of free and immobilized laccase were determined by measuring the laccase activity using ABTS as substrate over a 0.1–1.5 mM range of initial concentrations. The parameter values were obtained by non-linear curve fitting of the plot of reaction rate versus substrate concentration using the MicroCal Origin software v.3.01 (SPSS Inc., Northampton, USA).

2.7. Degradation of reactive dyes by immobilized laccase

A solution of single reactive dye (50 mg/L) (RB5, RB114, RR180, RR239, RY15 or RY176) or a mixture of three dyes 50 mg/L (RB5, RY15 and RR239) was continuously orbital stirred (240 rpm) with 0.4 g of support immobilized with laccase (0.067 g/mL of laccase per g support) in phosphate buffer solution pH 7.0, final volume of 25 mL at 35 °C. Three cycles of dye degradation were carried out. Between each cycle, the support was washed three times with phosphate buffer pH 7.0, 100 mM (±100 mL each wash). Dye decolourization was determined by monitoring the decrease in the absorbance peak at the maximum wavelength for each dye: RY15 (416 nm), RR239 (542 nm), RB114 (593 nm), RB5 (579 nm), RR180 (540 nm) and RY176 (421 nm). UV-visible spectrophotometer (Thermo, model UV1) was used in all experiments. Decolourization is reported as: % decolourization = $(A_i - A_f)/A_i \times 100$, where A_i is the initial absorbance or total area from the initial spectrum and A_f is the final absorbance or total area from the final spectrum. A control with support alone (without enzyme immobilization) was carried out at the same conditions in order to determine the dye adsorption by the support. For each assay duplicate runs were made.

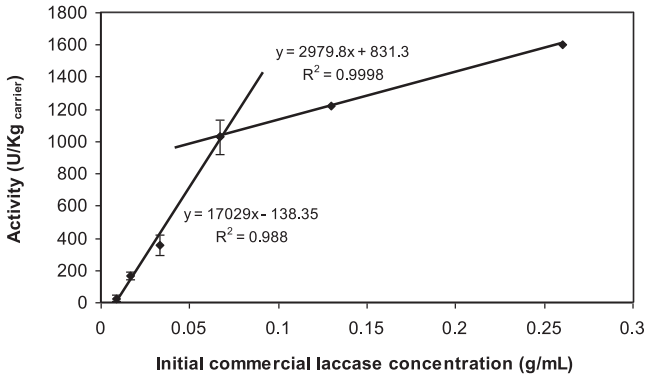

Fig. 1. Influence of initial enzyme concentration on the activity of the immobilized commercial laccase on coconut fiber by adsorption at pH 7.0 and 3 h 30 min of contact time. Due to the limited number of experimental points, the fit lines are approximate relationships.

3. Results and discussion

3.1. Immobilization of commercial laccase on green coconut fiber by adsorption

The aim of this study was to immobilize commercial laccase using a simple, effective and inexpensive process for the degradation of reactive textile dyes. The effect of enzyme concentration, pH and contact time between enzyme solution and coconut fiber on the activity of immobilized commercial laccase was studied and optimized.

In order to determine the optimum commercial laccase concentration to be immobilized, several experiments with the same amount of carrier and buffer were performed with initial enzyme concentrations ranging from 0.008 to 0.26 g/mL at room temperature. Contact times between the enzyme and the coconut fiber and pH were set at 3 h 30 min and 7.0, respectively. The results are presented in Fig. 1 (due to the reduced limited number of experimental points, the fit lines are approximate relationships). It can be seen that the activity of the immobilized laccase increases with the enzyme concentration, however not always in a linear way. Two slopes are observed, with the first one, for lower enzyme concentrations until 0.067 g/mL, steeper than the second one. According to the literature [21,22] enzyme adsorption is not restricted to a monolayer on the support, and adsorption of secondary layers has been reported. Thus, the first slope possibly corresponds to the formation of an monolayer of enzyme, while the second slope corresponds to enzymes binding on top of the monolayer. A control experiment with fiber without enzyme was also performed and no activity was found when only the fiber was used as catalyst.

Recovered activity and immobilization yield were calculated, and results are listed in Table 1. Recovered activity was maximum at the change of slope (0.067 g/mL), from which it begins to decrease (in a non-regular way). The maximum recovered activity at this point suggests that probably at this concentration the enzyme molecules are immobilized at close proximity to each other covering the entire support surface, thus preventing the enzyme deactivation. In other words, when enzyme load was increased,

Table 1
Influence of initial enzyme concentration on recovered activity and immobilization yield and their respective standard deviation.

Enzyme concentration (g/mL)	Recovered activity (%)	Immobilization yield (%)
0.008	11 ± 1	3.3 ± 0.5
0.017	4.2 ± 0.2	43 ± 3
0.033	6.7 ± 0.1	45 ± 1
0.067	12 ± 2	38 ± 9
0.13	8 ± 2	38 ± 5
0.26	9.6 ± 0.7	23 ± 4

Table 2
Influence of pH of the solution on recovered activity and immobilization yield and their respective standard deviation.

pH	Recovered activity (%)	Immobilization yield (%)
4.0	31.0 ± 0.1	11 ± 1
5.0	45.7 ± 0.2	13 ± 3
6.0	24.5 ± 0.2	17 ± 2
7.0	12.0 ± 0.1	38 ± 5
8.0	18.2 ± 0.3	20 ± 1
9.0	14.0 ± 0.4	29 ± 2

more enzyme molecules were immobilized and less area on the support is available for laccase to spread itself, which may prevent loss in activity [23]. Above this concentration, probably a second layer of commercial laccase was adsorbed on the first layer. Although more molecules were immobilized on coconut fiber, not all of them were available to the substrate, causing decrease in recovered activity. Coconut fiber surface does not have a porous structure, and it has a low surface area [24]. This poor surface area limits the number of enzyme molecules to be immobilized, facilitating the multilayer adsorption of enzymes [25]. Immobilization yield increases until an enzyme concentration of 0.033 g/mL, point from which starts a slow decrease (in a more regular way). With these results and taking into account both values of recovered activity and immobilization yield, the commercial laccase concentration of 0.067 g/mL was selected to continue the studies, since it is the concentration that offers the best relationship among the values of recovered activity and immobilization yield. From this enzyme concentration (where the slope changes), no increase in recovered activity or immobilization yield is observed, so there is no point on using higher initial enzyme concentrations.

The effect of pH for ABTS oxidation with immobilized laccase was studied at values from 4.0 to 9.0. Enzyme concentration and contact time between the support and the enzyme solution were set at 0.067 g/mL and 3 h 30 min, respectively. The pH profiles of the free and the immobilized laccase are shown in Fig. 2. It can be clearly observed from Fig. 2(b) that a maximum of immobilized enzyme activity was reached at pH 7.0–8.0 (and for values lower or higher than 7 the activity decreases). This optimum range is very similar to that observed for the soluble laccase (Fig. 2(a)) indicating that the immobilization procedure on green coconut fiber does not affect the enzyme charge, which has also been reported in other studies [26]. Subtle changes in the activity/pH profiles have been reported on the immobilization of enzymes to charged supports [27].

Immobilization parameters, recovered activity and immobilization yield, were calculated for all the range of pH studied. Table 2 shows that immobilization yield and recovered activity were dependent on the pH of adsorption. Interactions between the molecule and its environment influence the structure of an enzyme molecule and these interactions are pH-dependent [28]. The best result for recovered activity (45.7%) was achieved at pH 5.0, value from which it starts to decrease. On the other hand, immobilization at pH 7.0 significantly improved the amount of immobilized enzyme (37.5%). The commercial laccase immobilization at pH 5.0 results in a high decrease in the immobilization yield, as shown in Table 2. Thus, pH 7.0 was chosen to continue the studies of optimization, as it represents the value where more enzyme was immobilized and, despite of having a lower recovered activity, it corresponds to the highest activity of immobilized enzyme (Fig. 2(b)).

Finally, the influence of contact time between coconut fiber and commercial laccase solution was evaluated at pH 7.0 and two different enzyme concentrations: 0.033 g/mL and 0.067 g/mL (data not shown). Similar contact time profiles were achieved for both enzyme concentrations studied. It was observed that commercial laccase immobilized activity increases as time increased until 3 h 30 min. From 3 h 30 min till 5/6 h no significant changes in immobilized laccase activity were observed, but after this time the activity tends to fall. Probably for longer contact times some enzyme desorption takes place or the enzyme starts to adsorb at the second monolayer of enzyme, thus ceasing the availability of the enzymes to react with the substrate. These results can be confirmed by the recovered activity and immobilization yield results presented in Table 3. In both cases immobilization yield increased with the increase of contact time once more till 5/6 h, showing that there is no need to leave the support and the enzyme solution in contact for longer periods. By the recovered activity that increases till 3 h 30 min and then starts to decrease, it is possible to conclude that there is no advantage in leaving the enzyme solution in contact with the coconut fiber for longer times, since it does not promote

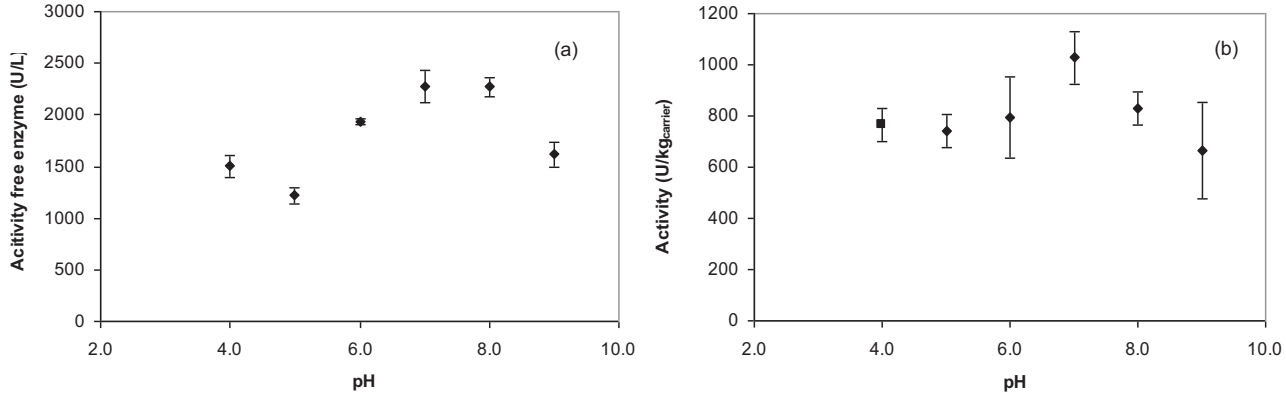

Fig. 2. Effect of pH of the solution on: (a) initial activity free laccase and (b) activity of the immobilized commercial laccase on coconut fiber by adsorption at 0.067 g/mL of enzyme and 3 h 30 min of contact time of immobilization.

Table 3
Influence of contact time on recovered activity and immobilization yield for 0.033 g/mL and 0.067 g/mL of enzyme and their respective standard deviation.

Contact time (h)	Recovered activity (%)		Immobilization yield (%)	
	0.033 g/mL	0.067 g/mL	0.033 g/mL	0.067 g/mL
0.5	5 ± 1	4.4 ± 0.2	38 ± 6	32 ± 1
1.0	7 ± 3	3.8 ± 0.1	36 ± 14	37 ± 1
2.0	4 ± 1	3.3 ± 0.3	57 ± 10	45 ± 9
3.5	5 ± 2	3.5 ± 0.3	44 ± 12	40 ± 4
5.0	4 ± 1	2.6 ± 0.1	69 ± 9	55 ± 1
6.0	5 ± 1	2.2 ± 0.2	51 ± 19	66 ± 3
7.0	3 ± 1	1.6 ± 0.6	75 ± 5	60 ± 1
8.0	3 ± 1	3.5 ± 0.7	71 ± 22	32 ± 6

the immobilization. Although more molecules were immobilized on coconut fiber, not all of them were available to the substrate, causing a decrease in recovered activity. So, this is a way to avoid the formation of the second monolayer and the loss of enzyme activity.

3.2. Immobilized enzyme properties

3.2.1. Thermal stability

The thermal stability is one of the most important features concerning the application of the biocatalyst. Enzyme immobilization often limits its freedom to undergo drastic conformational changes and thus results in increased stability towards denaturation [27].

Thermal stability experiments were carried out with free and immobilized enzyme, which were incubated at 60 °C. The immobilized enzyme was obtained by contacting 0.067 g/mL of a commercial laccase solution of pH 7.0 with the coconut fiber for 3 h 30 min. The thermal deactivation model (Eq. (1)) was fitted to experimental data (Fig. 3) and the model parameters are listed in Table 4, for free and immobilized enzyme. As it can be seen from the results in Table 4, the α values are quite small, indicating that possibly these can be discarded. Therefore it is possible to use a simple exponential decay model, that states the final activity of the enzyme as zero, as reported by many authors, such as: Longo and Combes [29] and Santos et al. [30]. However, the final activity of the enzyme observed is very small but not zero, then the correct formula, used in this work, is the Eq. (1).

Fig. 3 shows that commercial laccase immobilization led to a significant stabilizing effect towards heat denaturation at 60 °C. The immobilized laccase was inactivated at a much slower rate than that of the free form under the same conditions. After 5 min of incubation at 60 °C the free enzyme shows the same deactivation that the immobilized one presents only after 1 h of incubation. These results can be confirmed by the kinetic parameters of thermal deactivation presented in Table 4. The half-life time ($t_{1/2}$) is the period of time that takes for a substance undergoing decay to decrease by half. The free enzyme has a half-life of 0.02 h whereas the value for the immobilized one is 0.14 h, 6.6 times higher. This 6.6 value is exactly the stabilization factor (F) which shows that the immobilization of commercial laccase on coconut fiber promoted an improvement on thermal stability, as immobilized commercial laccase is more stable than the free one at 60 °C. The increased stability of immobilized laccase was due to the restricted conformational mobility of the molecules following immobilization. Other authors obtained similar results when immobilizing laccase on other supports [31]. The increased resistance to thermal denaturation of laccase arising from immobilization would be an advantage for its industrial application due to the high temperatures used in the industrial processes [32].

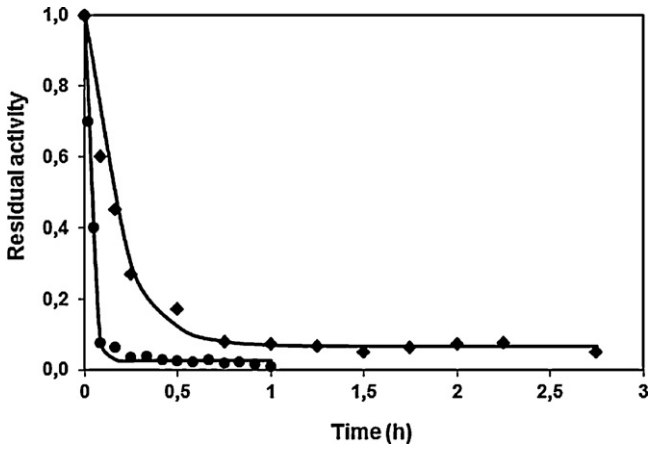

Fig. 3. Thermal stability at 60 °C of (●) free and (♦) immobilized commercial laccase obtained by adsorption at pH 7.0; 0.067 g/mL of initial laccase concentration and 3 h 30 min of contact time.

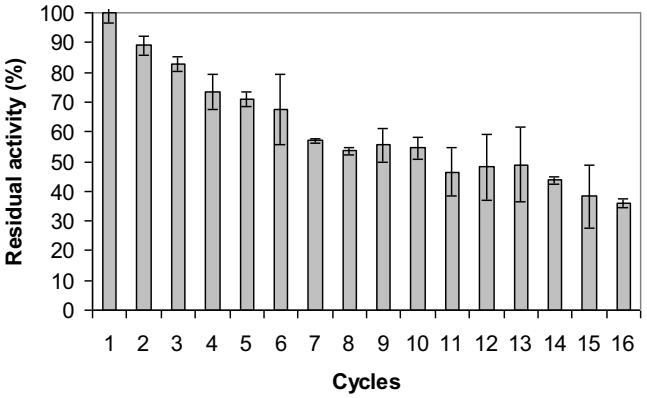

Fig. 4. Operational stability of commercial laccase immobilized on coconut fiber by adsorption at pH 7.0; 0.067 g/mL of initial laccase concentration and 3 h 30 min of contact time.

3.2.2. Operational stability

The reusability of the commercial laccase immobilized on coconut fiber by adsorption was studied by cycles of ABTS oxidation due to its importance for industry to reduce the processing costs. From the results shown in Fig. 4, it was possible to observe that, despite only (weak) physical bonds were involved, the operational stability of this system was good, losing only about 30% of the initial enzyme activity after 5 cycles of reaction and 45% after 13 cycles. After that, little activity loss was determined. Successful reuse of various immobilized laccase systems has been reported by other investigators [33]. Some of these works present better operational stabilities than the verified in our study due to the immobilization method used. The physical adsorption is known for having only weak bonds involved and, probably, the most pronounced loss of activity is due to enzyme leaching during washings. Nevertheless, the achieved values are good results and commercial laccase immobilized in coconut fiber by adsorption can be considered as a good choice of insoluble biocatalyst to be used in continuous reactions studies.

3.2.3. Kinetic properties

The free and the immobilized commercial laccase exhibited a normal behaviour of an enzyme reaction with ABTS as substrate. Thus, the kinetic parameters were calculated according to the classical Michaelis–Menten equation [34].

The Michaelis–Menten parameters v_{max} and K_M were obtained by non-linear fitting of reaction rate versus substrate concentration using the MicroCal Origin software v.3.01 (SPSS Inc., Northampton, USA). It was tried to use the same initial enzyme activities in both

Table 4
Kinetic parameters of thermal deactivation at 60 °C and Michaelis–Menten constants of free commercial laccase and immobilized by adsorption on coconut fiber at pH 7.0; 0.067 g/mL of initial laccase concentration and 3 h 30 min of contact time.

Enzyme	Thermal parameters					Michaelis–Menten parameters	
	k (h^{-1})	α	$t_{1/2}$ (h)	F	R^2	K_M (mM)	v_{max} (mM/min)
Free	34.8	0.026	0.02	1.0	0.998	0.0044	0.024
Immobilized	5.64	0.067	0.14	6.6	0.995	0.0501	0.133

Table 5
Dye mixture degradation (%) by immobilized laccase based on the absorbance peak at maximum absorbance wavelength of each dye present in the mixture and on the area under all dye absorbance spectrum range. It was taken into account the percentage of adsorption and of enzymatic activity.

λ (nm)	Total dye degradation (%)	Adsorption by the coconut fiber (%)	Dye degradation by the immobilized enzyme (%)
416	58	13	45
542	51	1.7	49
579	79	16	63
Area under all spectrum	54	0	54

studied cases. Table 4 shows the kinetic constants determined and Fig. 5 shows the initial reaction rate of increasing concentrations of ABTS for free and immobilized commercial laccase. The solid line represents the fit of Michaelis–Menten model to experimental data. K_M value is most useful in proving the ability of an enzyme to bind its substrate [35]. The lesser the value of K_M, the higher is the affinity for the substrate. An increase in the K_M value for the reaction of ABTS with the immobilized laccase was observed. The same results have been reported by other researchers [26], indicating a lower affinity for the substrate caused by diffusional limitations and decreased enzyme flexibility after immobilization. Moreover, diffusional limitations are less significant in coconut fiber, as immobilization occurs on the surface. Diffusional substrate limitations [33], substrate partitioning, protein conformational changes [36] and decreased protein flexibility [37] have been associated with the decrease in the enzymatic properties after immobilization. However, the v_{max} value of the immobilized enzyme increased compared to the free enzyme, probably because of the adsorption by the support: the local concentration increases, increasing the reaction rate.

3.3. Dye decolourization by immobilized laccase

Decolourization of RB5, RB114, RR180, RR239, RY176 and RY15 were achieved in a batch reactor with commercial laccase immobilized on coconut fiber for three cycles of reaction. Information about these dyes and the optimum pH (between 5.0 and 7.0) for dye degradation is available in Tavares et al. [6]. A high decolourization percentage of RB5, RB114 and RY15 were obtained in the first cycle, 90%, 90% and 80%, respectively. For RR180 and RR239, a moderate degradation of 30% was observed. No significant reduction of colour was observed for RY176. The second cycle presented a good degradation for both RB5 (80%) and RB114 (62%), while RY15 shows a significant decrease on the decolourization (39%). A poor degradation for the third cycle was observed. This tendency can be explained, when compared to ABTS oxidation cycles (Fig. 5), by the laccase mediator. It is known that no mediator is necessary for ABTS reaction with laccase. However, for most dyes and other phenolic compounds, a mediator is necessary to complete the catalytic cycle with laccase [9,38]. The laccase used in this study is a commercial formulation which includes a mediator. The mediator probably is not being reoxidised, as occurs in a system with free enzyme. Thus, after some time there is no longer available mediator to dyes oxidation. Additionally, the difference of dye decolourization percentage among the different dyes can be explained mainly due to the different molecular structure of the dyes and different potential redox as observed in an earlier study with free laccase [38].

When immobilized enzymes are used in decolourization studies the evaluation of the adsorption capacity of the support is necessary. Generally, the colour removal is the result of both enzymatic and adsorption processes. When an operation is carried out batchwise, the overall extent of decolourization is strongly affected by the physical removal of the dye from the liquid phase. Thus, another point that should be considered in this study is the colour removal

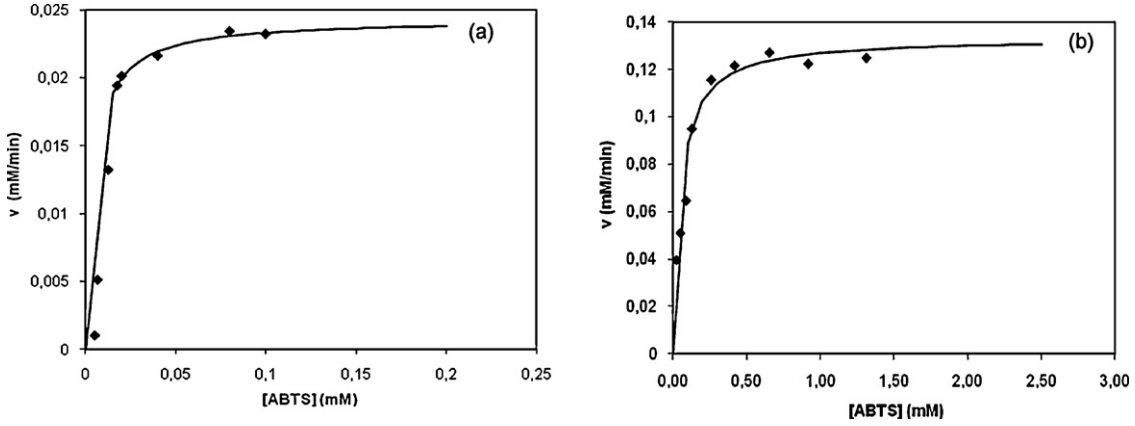

Fig. 5. Initial reaction rates for different concentrations of ABTS (a) with free and (b) with immobilized commercial laccase on coconut fiber at pH 7.0; 0.067 g/mL of initial laccase concentration and 3 h 30 min of contact time. The solid line represents the fit of Michaelis–Menten model to experimental data.

by adsorption on the support. Many dye decolourization tests carried out with immobilized laccase have showed that support adsorption presents a significant contribution to the colour degradation [14,15]. In order to verify this tendency, a control experiment with dyes and support alone was carried out. The adsorption of the dye by the support surface was around 40% for RB114, 48% for RY15, 23% for RB5, 4% for RY176, 7% for RR180 and no adsorption for RR239 was detected. These results show that, depending on the dye, in the first cycle of the reaction with immobilized laccase, a percentage of colour removal was due to adsorption of the dyes by the support. In the next cycles, partial saturation of the support occurred and the contribution of laccase increased. Using the supported laccase in experiments that involve the consecutive addition of several dye samples the catalytic effect of the enzyme is evidenced, maybe due to the progressive saturation of the active free-sites of the support. This was observed by Peralta-Zamora et al. [14] applying consecutive feeds to a batch system loaded with immobilized laccase. Since the enzyme is only active in the presence of a mediator, they observed that, in the absence of the mediator, the extent of decolourization in the consecutive dye additions decreased as adsorption approached equilibrium; on the contrary, in the presence of the mediator, the extent of decolourization during each cycle remained constant up to eight dye additions. Comparing dyes degradation with the immobilized system (90% for RB5, 90% for RB114, 93% for RY15, 96% for RR239, 93% for RR180 and 0% for RY176) and with free laccase [34,39], at the same conditions of dye concentration, temperature and agitation, a considerable difference was observed. A decreased in all dyes degradation was observed taking into account only the degradation by the immobilized laccase. This might be attributed to the diffusional limitations and the loss of laccase activity during the immobilization process.

As most industrial effluents contain a mixture of dyes, this part of the study is important in order to validate the applicability of the process. To our knowledge there is no study on degradation of a mixture of dyes by an immobilized laccase. Table 5 shows the decolourization of a mixture of three dyes, RY15, RR239 and RB5, by immobilized laccase and by adsorption. The degradation of the mixture was obtained from both wavelength corresponding of each dye and area integration of the all visible spectrum. When compared with a single dye, a decrease in dye degradation of the mixture was observed. This probably occurs due to the competition between several compounds for the active sites of the enzyme. Comparing the decolouration with free laccase [40], at the same conditions of dye concentration, temperature and agitation, a considerable difference was also observed, a decrease in total decolouration of the mixture about 36%, from 90% (free enzyme) to 54% (immobilized enzyme), was obtained with immobilized enzyme. This might be also attributed to the diffusional limitations and the loss of laccase activity during the immobilization process.

4. Conclusions

Green coconut fiber was successfully used to immobilize commercial laccase by adsorption in the optimized conditions, being a simple and inexpensive method with an easily available support. A high decolourization percentage of practically all reactive textile dyes in the first two cycles and a good dye mixture decolourization by the immobilized enzyme were obtained. The immobilization allowed enzyme reuse (with high operational stabilities) and has been shown to improve its stability, which could be a potential advantage in wastewater treatment.

This work may provide a basis for the development of suitable biocatalysts for continuous colour removal from various industrial effluents at large scale.

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