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Reactive dyes and textile effluent decolorization by a mediator system of salt-tolerant laccase from *Peniophora cinerea*

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ABSTRACT

Peniophora cinerea is a white-rot fungus with ability to decolorize dyes in saline textile effluent. After cultivation in a medium composed by sucrose, corn steep liquor, copper, and other minor components, *P. cinerea* produced 1000 U/L of laccase, but no lignin peroxidase or manganese peroxidase. The produced laccase was purified by precipitation with ammonium sulfate (80%) and anion exchange chromatography. Isoelectric focusing revealed a total of eight salt tolerant laccase isoenzymes with p*I* between 3 and 6, and molecular weights in the range of 26.2 and 72.6 kDa. Five compounds were then tested and compared for their ability to act as mediators in the decolorization of the reactive blue 19 dye by these laccases of *P. cinerea*. Syringaldehyde was the best mediator since it increased in almost 3-fold the dye decolorization when compared to the decolorization by using laccase alone. Addition of Mn^{2+} and oxalate to this system increased in 4.8-fold the initial decolorization rate (178 μmol/L min). When applied for real effluent decolorization, this system promoted the highest decolorization after 72 h at 50 °C and pH 4. This laccase-mediator system was considered promissory for application on the treatment of industrial dye effluents.

1. Introduction

Reactive dyes are colored compounds that contain functional groups able to form covalent bonds with active sites in fibers, and that offer improved wash fastness over direct dyes. Unfortunately, fiber fixation is always followed by alkali-induced dye hydrolysis, leading to molecules that do not undergo covalent bonding with cellulose. Moreover, the side reactions requires large amount of salt for high-quality exhaustion, generating problems for conventional treatments. Therefore, textile effluents are highly colored and saline, contain non-biodegradable compounds, and are towering in biochemical and chemical oxygen demand [1,2].

Laccase (benzenediol oxygen oxidoreductase, EC 1.10.3.2) is a versatile polyphenol oxidase that oxidizes organic compounds and transition metals. The one-electron oxidation of these reducing substrates occurs concomitantly with a four-electron reduction of molecular oxygen to water. Laccases produced by wood-rotting fungi play an important role in the degradation of lignin and xenobiotics, mainly if associated to mediators or low molecular weight compounds with ability to chelate and reduce Fe^{3+}. The mediators for laccases include N-heterocyclics bearing NOH-groups, phenolic compounds, and manganese ions [3–6]. Some laccase mediator systems have been used for pulp biobleaching, decolorization and detoxification of effluents, and synthesis of chemicals [7–10].

Laccases are able to oxide Mn^{+2} to Mn^{+3} in the presence of oxalate or malonate [4]. The resulting Mn^{3+} causes decomposition of organic molecules, formation of superoxide radicals, and subsequent reduction of superoxide to H_2O_2. All these reactions may contribute to an increased substrate oxidation rate [11]. The electron-withdrawing strength of manganic chelates can be greatly modulated. Free (hexaquo) Mn^{3+} ions are 1.52-eV electropositive with respect to Mn^{2+} ions. The nature and concentration of the chelator determine (i) the readiness by which the Mn^{3+} chelate is formed, (ii) the half-life of the chelate, and (iii) its substrate range and reaction rate [12]. These findings were experimentally verified with laccases from *Stropharia rugosoannulata* [11]. According to Archibald and Roy [12] phenoxy and other aryloxy radicals, as well as O_2^-, can readily generate Mn^{3+} from Mn^{2+} chelates with laccase of *Trametes versicolor*. A system comprising laccase and 4-hydroxybenzoic acid (HBA) or synthetic lignin exhaustively oxidized linoleic acid, and the peroxidation rate was greatly enhanced by Mn^{2+}, which was oxidized to Mn^{3+} by laccase/HBA [13].

Peniophora cinerea is a white-rot fungus with ability to decolorize dyes in medium containing NaCl and saline effluent [14].

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Laccases produced by this fungal strain have shown salt tolerance, which is an interesting feature for biotechnological applications, since many processes occur in high ionic strength, such as the decolorization of textile effluents. Our interest in laccases produced by *P. cinerea* stemmed from our previous investigations on treatments of wastewaters from textile industry, which are extremely resistant to microbial attack. In the current study, several compounds were tested and compared for their ability to act as mediators in the decolorization of the reactive blue 19 dye by laccases of *P. cinerea*. The decolorization was further investigated with respect to the effects of laccase-mediated Mn^{+3} generation, and monitored by the decolorization activity of dyes and textile effluent.

2. Experimental

2.1. Chemicals

2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonate (ABTS), 4-hydroxybenzoic acid (HBA), 3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde), 1-hydroxybenzotriazole (HBT), 3, 4-dihydroxiphenilacetic acid (DOPAC), 2,3-dihydroxybenzoic acid (2,3 DHBA), linoleic acid, oxalic acid, and reactive blue 19 (Remazol Brilliant Blue R) were obtained from Sigma–Aldrich. Reactive red 271 (Cibacron Red FN-2BL; λ_{max} 525 nm, reactive group monochlorotriazine) was supplied by a textile industry (São Paulo, Brazil). Other chemicals were all of analytical grade. Buffers and solutions were prepared in Milli-Q ultrapure water.

2.2. Textile effluent

The industrial effluent used in this study was supplied by a textile industry (São Paulo, Brazil) that uses different dyes and chemicals including detergents, salts and surfactants. The amount of these chemicals in the effluent varies according to the industrial process utilized, and therefore, the exact composition of the effluent is unknown. The pH and conductivity values of this effluent were 12.6 and 11.3 mS/cm, respectively.

2.3. Fungal strain and culture conditions

P. cinerea CCB204 was isolated from Restinga forest (coastal ecosystem). Fungal inoculum was prepared from mycelia grown on 2% (w/v) potato dextrose agar (PDA) at 28 °C during 7 days, under static conditions. The liquid culture medium (50 mL) containing sucrose (5 g/L), corn steep liquor (0.5% v/v), KH_2PO_4(0.2 g/L), $MgSO_4{\cdot}7H_2O$ (0.05 g/L), and $CaCl_2{\cdot}2H_2O$ (0.013 g/L) was sterilized at 121 °C for 15 min and inoculated with three mycelia PDA plugs (∅ 7 mm) in 250-mL Erlenmeyer flasks, which were maintained at 25 °C under static conditions, during 20 days, to obtain the maximum production of laccase. In the fourth day of cultivation, copper sulfate (1 mM) was added to the medium to induce the production of laccase.

2.4. Laccases purification

After 20 days of cultivation, 100 mL of the liquid culture (two flasks) was harvest, filtered through 0.45 μm membrane, and assayed for enzymatic activity. Two purification processes were then carried out. Initially, the suspension was applied on a DEAE-Sepharose CL-6B anion exchange column (1.5 × 12 cm) pre-equilibrated with 10 mM phosphate buffer (pH 8.0), and fractions of 5.0 mL were collected at a flow rate of 0.25 mL/min (preparative purification). Bound proteins were eluted with 25 mL of 0.2 M NaCl. Fractions corresponding to the laccase activity were collected, pooled, concentrated, diafiltrated (3 kDa cut-off, Millipore), and stored at −10 °C for later use in dye decolorization assays. Alternatively, the extract was concentrated by using ammonium sulfate (at 80% saturation) and was loaded onto a DEAE-Sepharose CL 6B column at the same conditions previously described. This step had as objective to characterize the laccase isoenzymes (analytical purification). The proteins were eluted using 20 mL of NaCl solutions each one in the following concentration (M): 0.05, 0.075, 0.1, and 0.2. Fractions with laccase activity were loaded onto a Mono-Q column (6.4 × 30 mm). Laccase was eluted with a linear gradient of 0–0.2 M NaCl in 10 mM sodium acetate solution (pH 4.7 or 5.8) at a flow rate of 0.5 mL/min. All the chromatography steps were performed using a FPLC (fast protein liquid chromatography) system (AKTA) and the elution was followed between 280 and 600 nm of absorbance. Throughout the purification process, fractions were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) for laccase activity and isoelectric focusing (IEF) [15].

2.5. Spectrophotometric determinations

Laccase activity was determined by oxidation of 0.1 mM ABTS (ε_{420} = 36,000 M/cm) at pH 4.0 [16]. Manganese peroxidase and lignin peroxidase activities were monitored by oxidation of red phenol [17] and veratryl alcohol [18], respectively. The concentration of Mn^{3+}-oxalate (ε_{270} = 5.5 mM/cm) was determined at 270 nm [19]. $MnSO_4$ 1 mM and sodium oxalate 100 mM were applied with 1 U/mL of laccase at pH 4.5. All the determinations were carried out at 25 °C in a double-beam spectrophotometer Hitachi model U-2900.

2.6. Laccase characterization

SDS–PAGE analysis of proteins was performed on 12% polyacrylamide gel [15] and proteins were stained with silver nitrate (GE Healthcare). Native-PAGE was performed by incubating the gel at 25 °C in sodium acetate pH 4.5 (0.1 M) containing 1 mM ABTS. The molecular weight of denatured laccase was estimated by SDS–PAGE. p*Í*s for laccases isozymes were determined using a Phast system isoelectric focusing (IEF) unit (GE Healthcare) on a 55 mm pH 3–9 linear immobiline strip. The pH profile was determined at 25 °C under various pH values varying between 2.5 and 4.5 with citrate–phosphate buffer, and between 4.5 and 5.5 with sodium-acetate buffer. The Michaelis–Menten coefficient (*Km*) was determined using ABTS (0.05–50 μM) and the experimental data were analyzed according to the Lineweaver–Burk plots. The half-life (*t*½) of the enzyme, i.e., the time (h) required to attain 50% of loss in the enzymatic activity, was determined by incubating the enzyme at 50 °C for certain time intervals, and the residual activity was determined. The NaCl inhibition of laccase was assayed by the ABTS oxidation. The inhibition was quantified by the parameter I_{50}, the halide concentration at which 50% of the initial laccase activity was observed.

2.7. Decolorization assays

The decolorization of reactive blue 19 (RB 19) dye (140 μg/mL final concentration) was carried out in 1-mL cuvettes using 100 mM sodium-acetate buffer at pH 4.0 during 5 min at 25 °C, and was monitored at 592 nm (ε_{592nm} = 6170 M/cm). The decolorization activity was defined as the amount of enzyme required to decolorize 1 μmol of dye per minute. Screening for laccase mediators was based on the decolorization of RB 19 by pre-purified laccases of *P. cinerea* (1 U/mL final activity), in the presence of the compounds: syringaldehyde, HBT, HBA, DOPAC, and 2,3 DHBA, at

two concentrations (0.1 and 1 mM). RB was also decolorized by laccase alone as control for relative activity.

The effects of $MnSO_4$, sodium oxalate, and syringaldehyde on decolorization of RB 19 (140 μg/mL final concentration) were evaluated using 1 mM of each compound. The spectra UV–Vis of Reactive Red 271 (120 μg/mL final concentration) was determined using the catalytic system of lacase-syringaldehyde-Mn^{2+}-oxalate (LSMO) at the same conditions previously described.

2.8. Treatment of the textile effluent

For the textile effluent treatment, a reaction medium (5 ml) composed of 0.1 M sodium acetate (pH 4.0), 1.0 U/mL of pre-purified laccase, 1 mM syringaldehyde, 1 mM $MnSO_4$, 1 mM sodium oxalate, and the textile effluent (diluted 1:10), was prepared and maintained at 50 °C. The decolorization was expressed in percentage of absorbance decrease at 621 nm, after 72 h of reaction. Control assays were prepared in the absence of laccase.

3. Results and discussion

3.1. Purification and characterization of salt tolerant laccases

The extract containing laccase produced by *P. cinerea* was initially submitted to a preparative purification step to obtain an extract free of interfering and with high laccase activity. In this stage, the whole extracellular proteins produced by *P. cinerea* were loaded onto an anion exchange column and one peak containing laccase activity was eluted and collected in three fractions, 22, 23 and 24 (Fig. 1A). The main of these fractions achieved a specific activity of 8 U/mg of protein, with a purification factor of 16.7-fold, and yield of 67% (Table 1). SDS (Fig. 1B) and Native Page (Fig. 1C) analyses of the pre-purified fractions revealed several bands, most of them referred to laccase activity.

Since the preparative purification step revealed the presence of several laccase isozymes, an analytical purification step was then performed in order to characterize the enzymatic extract produced by *P. cinerea*. The extract was concentrated by ammonium sulfate and loaded onto the DEAE column and eluted with increased NaCl concentrations. Three peaks of laccase activity were eluted from this column (Fig. 2A). Peak 1 showed one band on SDS–Page correspondent to 72.6 kDa and with p*I* near to 6, showing a purified isoenzyme (Fig. 2B). Peak 2 was reloaded onto a Mono Q column pre-equilibrated at pH 5.8, and one band with p*I* near to 4 was separated from others with p*I* between 4.5 and 5.25 (Fig. 3A and C). The peak 3 was loaded into a Mono Q column pre-equilibrated at pH 4.7, and three peaks with laccase activity were eluted (Fig. 3B and C). Overall, *P. cinerea* produced eight laccase isoenzymes with different properties (Table 2). Laccases with similar p*Is* were not totally purified. Similar results were reported by Antorini et al. [20], who found two peaks with laccase activity after elution of extracts of *Pycnoporus cinnabarinus* on DEAE-Sepharose.

Laccase isoforms of *P. cinerea* showed differences in molecular weight, optimum pH, kinetic parameters, p*I*, and different degrees of inhibition by NaCl (Table 2). The inhibition by NaCl is attributed to the limited accessibility of the type 2/type 3 (T2/T3) copper atoms in the active site, where the reduction of molecular oxygen to water occurs. The halide binding to the T2/T3 trinuclear copper sites is different among various laccases [21,22]. Additionally, different laccases may have quite different tolerances toward inhibition by various halide species [23]. As the inhibition is mainly limited by the size of the putative channel that leads to the T2/T3 site, then the observed variation in I_{50} indicates a significant difference among the laccases produced by *P. cinerea*. While the plant *Rhus* laccase might have a "wide-open" channel leading to the T2/T3 site, fungal laccases seem to have T2/T3 channels with a defined

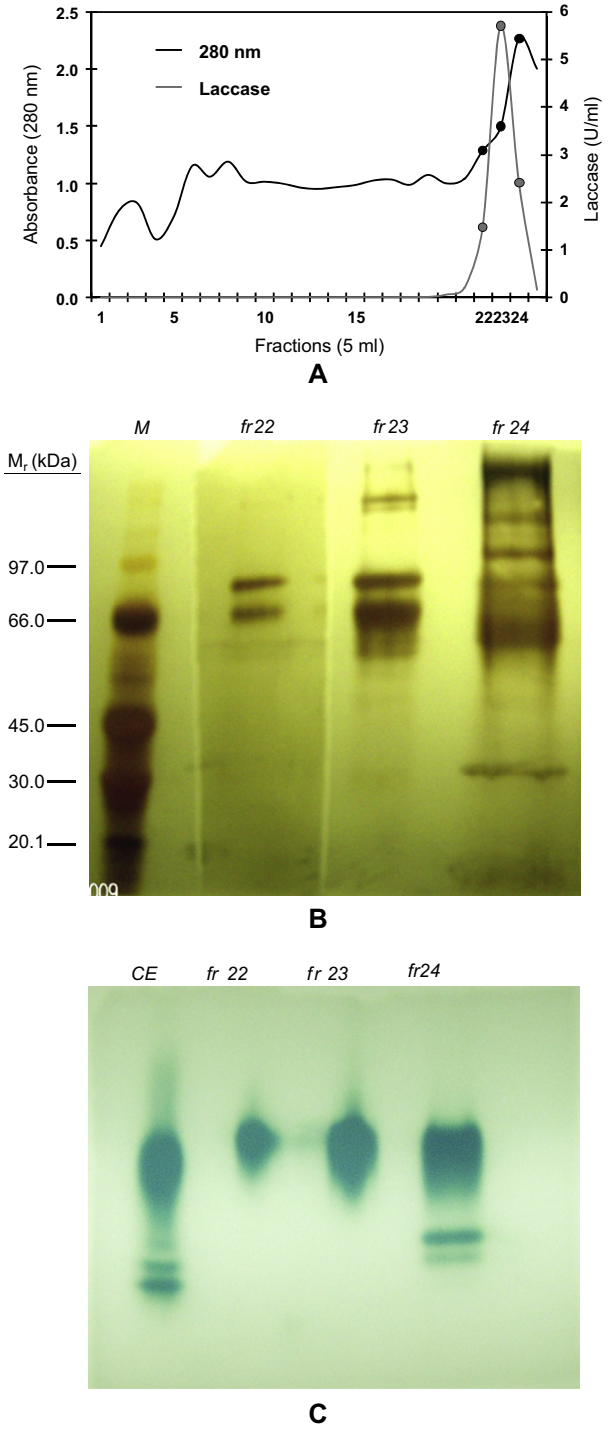

Fig. 1. Purification of laccases by anion exchange chromatography. (A) Elution of laccase (gray line) and 280 nm (black line) on a DEAE-Sepharose with a NaCl 0.2 M. (B) Silver nitrate-stained gels (SDS–PAGE) and (C) gels for laccase activity with ABTS 1 mM of samples from the fractions with laccase activity. *M* = Standard molecular mass markers (kDa), *fr* = fractions eluted from DEAE-Sepharose, *CE* = crude extract.

"cut-off diameter" that corresponds to the diameter of hydrated Cl^-. Laccases from *Sclerotium rolfsii* and *Coprinus cinereus* showed a NaCl I_{50} of 52 and 200 mM, respectively [24,25].

3.2. Laccases-mediator system for dye decolorization

Different compounds were evaluated as mediators for decolorization of the RB 19 dye by laccases of *P. cinerea* (Table 3). Among

Table 1
Characterization of the fractions with laccase activity after elution on DEAE-Sepharose.

Step	Activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	51	105	0.48	100	1
Fractions					
22	8	3.3	2.43	16	5.0
23	34	4.3	8.06	67	16.7
24	13	5.9	2.25	26	4.7

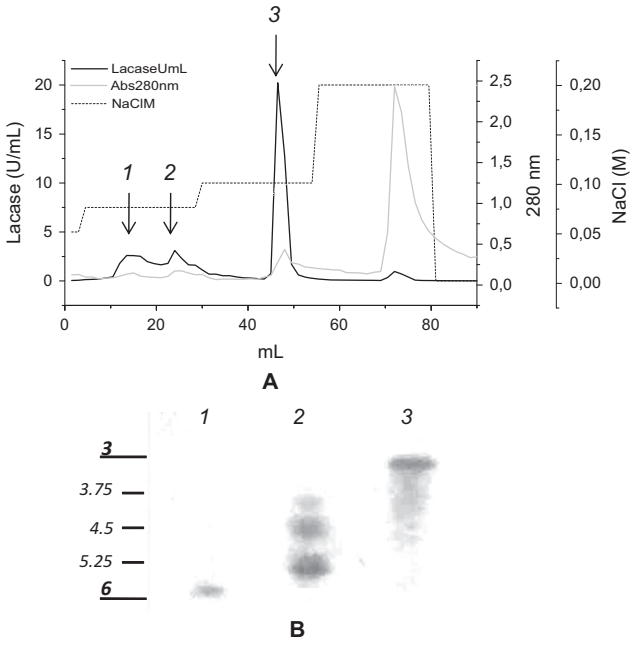

Fig. 2. Purification of laccases isoenzymes from *Peniophora cinerea*. (A) Laccase activity (U/mL) measured with ABTS (black line) and absorbance at 280 nm (gray line) from the three peaks detected when a gradient 0–0.2 M of NaCl (dashed line) was applied to DEAE-Sepharose column. Fractions (0.5 mL) were collected. (B) Native isoelectric focusing (IEF) from samples of the tree peaks with laccase activity eluted from the DEAE-Sepharose column. The gel was stained only with 1 mM ABTS. Lines 1, 2 and 3 correspond to peaks detected after elution on DEAE-Sepharose.

these compounds, syringaldehyde was the most suitable since it promoted a decolorization rate approximately three times higher than those obtained with HBT or HBA. On the other hand, no remarkable activity was detected when using DOPAC or 2,3 DHBA. These results are in agreement with other studies that have also found syringaldehyde as being more efficient than HBT for dye decolorization [26,27]. The effect of DOPAC and 2,3 DHBA on laccase activity showed a decrease not dependent on their concentration, so we can predict that they are not good substrates for laccases. However, as both DOPAC and 2,3 DHBA reduce Cu^{2+}, the decrease in activity was attributed to the formation of additional activated species. A redox cycling activity would be expected by continually reduce the metal and maintaining a high level of the reduced metal in the system and so, permitting laccase reactions to proceed slower [28].

Syringaldehyde and acetosyringone have been described as laccase natural mediators less toxic than synthetic mediators such as ABTS and HBT [29]. Despite HBT is considered an efficient laccase mediator for dye decolorization [30], biobleaching [31], delignification [32], and bioremediation [33], it is known that the oxidation of HBT generates a highly unstable intermediate, putatively an N–OH radical, which quickly decays into catalytically inactive secondary product(s), including benzotriazole [5,7].

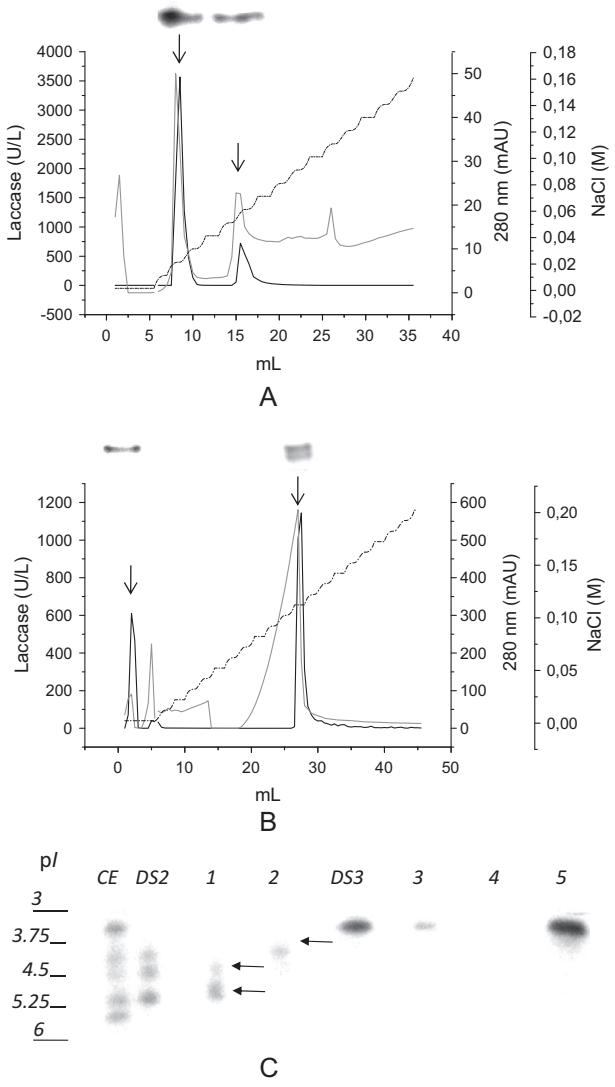

Fig. 3. (A) Laccase activity (U/L) measured with ABTS (black line) and absorbance at 280 nm (gray line) from the two peaks detected when a linear gradient 0–0.2 M of NaCl (dashed line) was applied to a Mono Q column equilibrated in sodium acetate buffer pH 5.8, or from the three peaks detected when a linear gradient 0–0.16 of NaCl was applied to a Mono Q column equilibrated in sodium acetate buffer pH 4.7. Fractions (0.3 mL) were collected. (B) Native 12% polyacrilamide gel electrophoresis of samples from different peaks with laccase activity is shown in site on the graphic. (C) Native IEF from samples of the five peaks with laccase activity eluted from the Mono Q column. The gel was stained only with 1 mM ABTS. Lines 1 and 2 correspond to peaks eluted from the Mono Q column equilibrated in pH 5.8; and Lines 3, 4 and 5 correspond to peaks eluted from the Mono Q column equilibrated in pH 4.7. *DS2* and *DS3* correspond to samples applied on Mono Q (peaks 2 and 3 obtained from DEAE-Sepharose); *CE* = crude extract.

In a subsequent step, a catalytic system consisting of laccase from *P. cinerea*, syringaldehyde, Mn^{2+}, and oxalate was used for RB 19 decolorization. This laccase-syringaldehyde-Mn-oxalate

Table 2
Biochemical properties of laccases isoenzymes produced by *Peniophora cinerea*.

Laccases	Molecular weight (kDa)	p*I*	Optimum pH	*t*1/2 50 °C (h)	*Km* (μM)[a]	I_{50} NaCl (mM)
Laccase A	72.6	6.0	4.0	19.4	12.7	421
Laccase B	72.0	4.0	3.0	<0.5	20.0	312
Laccase C	41.6	3.0	3.2	34.4	15.6	358
Laccases D and E	72.0	4.5, 5.25	2.6	11.9	–	–
Laccases F, G, H	32.4, 27.1, 26.2	3.0	2.6	8.3	–	–

[a] ABTS as substrate.

Table 3
Screening of the mediator used for decolorization of reactive blue 19 dye by laccase. The standard deviations were lower than 1% of the mean values.

Mediator	Mediator concentration (mM)			
	0.1		1.0	
	Activity (μmol/min L)	Relative activity (%)	Activity (μmol/min L)	Relative activity (%)
Control	36.47	100	36.47	100
HBT	39.71	109	39.71	108
HBA	35.66	98	52.19	143
Syringaldehyde	106.16	291	134.42	369
2,3 DHBA	1.62	4	10.53	29
DOPAC	4.86	13	29.98	82

(LSMO) system yielded a 4.8-fold higher decolorization activity (178 μmol/L min) than the control containing only laccase (Fig. 4A), showing an important improvement in the results. It is important to detach that laccases from *P. cinnerea* were able to directly oxidize Mn^{2+}, as revealed by the increase in the absorbance reading at 270 nm, corresponding to Mn^{3+}-oxalate formation (Fig. 4B). When using 1 mM chelator and lacase (1 U/mL), 53.1 μM were obtained for Mn^{3+}-oxalate that is similar to the data obtained by Schlosser and Höfer [11] using laccase of *S. rugosoannulata*. This is an important result because only few laccases are reported to directly oxidize Mn^{2+}, including laccases from *Pleurotus eryngii* [34] and *T. versicolor* [4].

In the presence of Mn^{2+}, the oxidation of different phenols and hydroquinones to the correspondent phenoxy radicals and semiquinones, catalyzed by laccase, may also lead to the formation of Mn^{3+}[4,12]. Probably, syringaldehyde phenoxy radical oxidizes Mn^{2+} to Mn^{3+}, which, together with *P. cinerea* laccase, increases the rate of dye decolorization. This system has a redox potential high enough to oxidize compounds usually not oxidized by laccase alone.

In a final step, decolorization assays using the Reactive Red 271 dye were performed in order to evaluate the applicability of the LSMO system in another dye. This dye was chosen because it is an azo dye, different from the previously used Reactive Blue, which is an anthraquinonic dye. The results revealed that the Reactive Red was not decolorized by laccase of *P. cinerea* alone; however, the dye was almost completely decolorized by the LSMO system in less than 2 min (Fig. 5).

In brief, both catalytic systems with laccase of *P. cinerea* (direct oxidation of Mn^{2+} and laccase mediator system with syringaldehyde) increased enzyme catalysis. However, the catalytic efficiency of the system for decolorization was dependent of the type of dye used. RB 19 (anthraquinonic dye) was decolorized faster in the system with syringaldehyde and Mn^{2+}, while for the Reactive Red (azoic dye) the reaction rate of decolorization by laccase was the same when using syringaldehyde or syringaldehyde with Mn^{2+}.

3.3. Textile effluent decolorization

The ability of laccases to decolorize simulated effluents and dyes has been documented in the literature, but few studies have shown the decolorization of real effluents. Among them, the most promising result was reported by laccase of *Trametes trogii* in the presence of HBT, which reached 60% of decolorization [35]. Other relevant data were reported by using commercial laccase

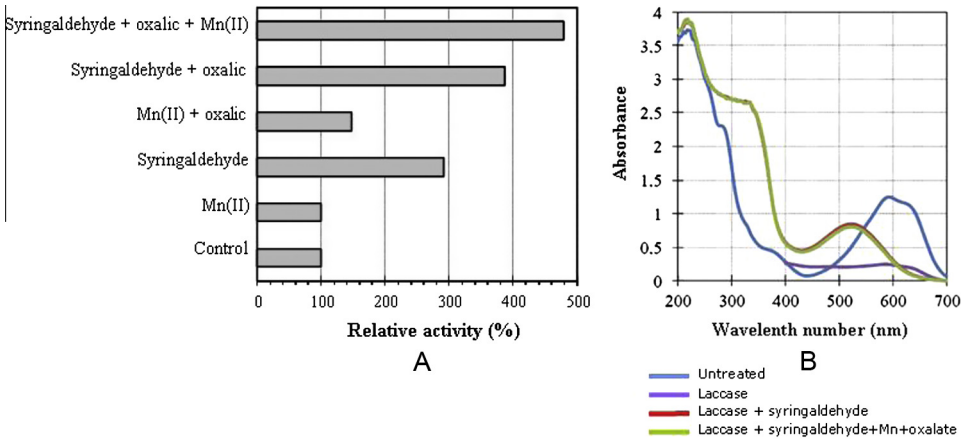

Fig. 4. (A) Decolorization of reactive blue 19 dye by laccases of *P. cinerea* in the presence of syringaldehyde, oxalic acid and Mn^{2+}. The standard errors were lower than 1% of the mean values. (B) Spectral scan (200–700 nm) for the decolorization of reactive blue 19 (100 mg/L).

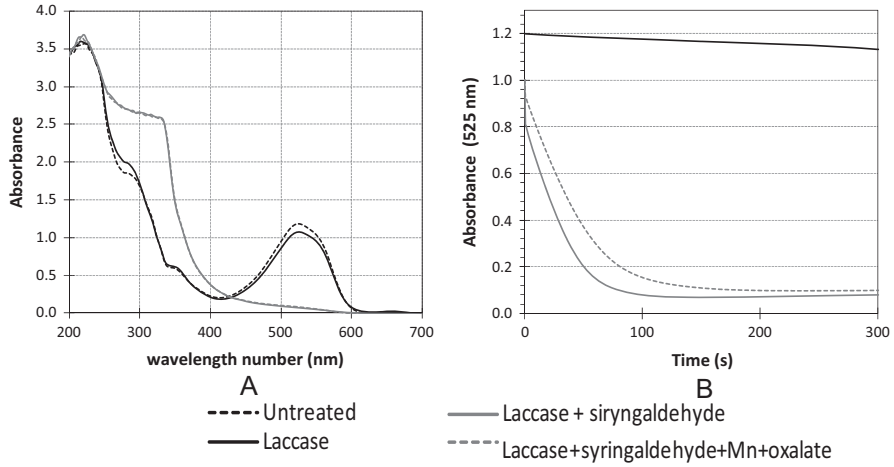

Fig. 5. (A) Reactive red decolorization by laccase-syringaldehyde-Mn-oxalatesystem. (B) Spectral scan (200–700 nm) for the decolorization of reactive red (100 mg/L).

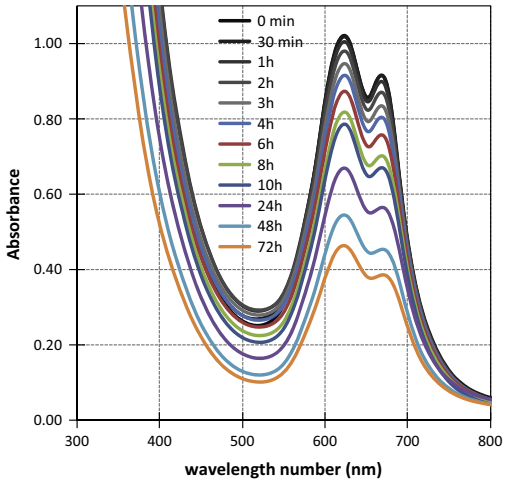

Fig. 6. Decolorization of textile effluent by laccase-syringaldehyde-Mn-oxalate system at different times.

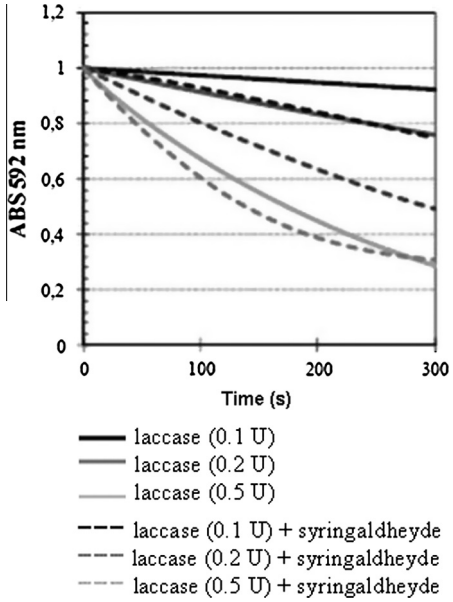

Fig. 7. Decolorization of textile effluent by laccase-syringaldehyde at different laccase concentration.

formulation (DeniLite® IIS) [36] and laccase from *Trametes pubescens* immobilized on alumina pellets [37]. In the present study, the treatment of a real effluent by the LSMO system with salt tolerant laccases of *P. cinerea* promoted 54.6% of decolorization after 72 h (Fig. 6). These results are very promising and reveal the high potential of the LSMO system for effluent decolorization.

The effluent decolorization when no Mn^{2+} and oxalate were added was determined at different laccase concentration. Decolorization increased with additional laccase and maximum decolorization of 77% was observed at 0.5 U laccase/mL (Fig. 7). Lower laccase dose was more efficient with addition of the mediator. This system could be used for textile wastewaters treatment, particularly as a polishing process for water recycling.

4. Conclusions

Many ligninolytic fungi are able to secrete laccases; however, few laccases are able to directly oxidize Mn^{2+}, as it was verified for the laccases of *P. cinerea*. Additionally, the present study greatly enhanced the catalyzes rate with salt tolerant laccases of *P. cinerea* in a system composed by syringaldehyde-oxalate-Mn^{2+}. This is the first evidence of salt tolerant laccase mediators with oxalate-Mn^{2+} acting on textile effluent decolorization, and this system can be considered a promising strategy for further biotechnological applications.

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