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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