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Full Length Research Paper

Purification and characterization of laccase from *Trametes hirsuta* Bm-2 and its contribution to dye and effluent decolorization

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In this study, the results demonstrated that *Trametes hirsuta* Bm-2 produced inducible laccases from wheat bran. Two laccase isozymes were partially purified by ammonium sulfate precipitation, anion-exchange chromatography and size-exclusion chromatography. The major laccase LacI, is a monomeric protein with apparent molecular mass of 65 kDa (SDS-PAGE). The optimal pH of the enzyme is 4 to 4.5 and the optimal temperature is 40 to 60°C with good stability up to 65°C. The *Km* values for non phenolic substrate 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and phenolic substrate 2,6-dimethoxyphenol (DMP) are 68 and 164 µM, respectively. Activity is increased by the addition of 1 mM Mn^{2+}, which is resistant to Ca^{2+}, Cu^{2+} and Cd^{2+} ions, partially resistant to EDTA and strongly inhibited by sodium azide, SDS and cysteine. Also, LacI is very resistant to ethanol and acetonitrile (20%), retaining 100% activity after 24 h incubation. Laccase was able to decolorize 100% dye acid blue and 36% textile effluent without any mediator addition, suggesting that it has the potential of been applied in bioremediation and synthesis of organic processes.

Key words: *Trametes hirsuta*, purification, laccases, effluent decoloration.

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2) are multicopper enzymes widely distributed in bacteria, yeasts and plants, they are mainly produced by white rot fungi, such as *Trametes versicolor, Pleurotus eryngii, Travetes villosa,* etc. (Morozova et al., 2007). These enzymes catalyze the oxidation of a wide range of phenolic and aromatic compounds by the removal of electrons until molecular oxygen is reduced to water (Thurston, 1994; Giardina et al., 2010). Laccases act upon monophenol, diphenol, metoxiphenol, polyphenol, aniline, benzenotiole, aryldiamines substrates and many others. Their range of action can extend to other substrates by the addition of small molecules, which act as mediators, to the reaction system (Kunamneni et al., 2008). Laccases have a diversity of biological functions, such as fungal development and morphogenesis (Leonowickz et al., 2001); they also control fungal pathogenicity (Zhu et al., 2001) and play an essential role in carbon recycling during lignin degradation (Kim et al., 2002). Interest in laccases has increased considerably over the last few decades, given the variety of biotechnological applications for these enzymes due to the wide range of substrates they can act upon. Laccases are useful in the removal of phenolic compounds from wine, and in beer stabilization (Minussi et al., 2002), paper pulp deliginification (Camarero et al., 2007) and in the development of fuel cells and biosensors (Ghindilis, 2000). In organic synthesis, they can transform functional groups or achieve the coupling of phenols and steroids (Ponsoni et al., 2007; Kunamneni et al., 2008), and they are also used in bioremediation processes for industrial dyes and effluents (Younes et al., 2007). The use of dyes has been increasing steadily in chemical, pharmaceutical,

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cosmetic and textile industries. The molecular structure of dyes is extremely diverse and this can result in genotoxic or cytotoxic effects, representing a high risk for both human health and the environment (Couto and Toca-Herrera, 2006). Laccases have been purified and characterized from many white rot fungi. These show heterogeneity in their structure and specificity of action, indicating that strains of the same genus, and even the same species, can produce different laccases, each one with its own unique characteristics (Baldrian, 2006). If we consider the diversity of applications for laccases, we must recognize the importance of identifying new sources of laccases with specific properties for a particular application. *Trametes hirsuta* Bm-2 is a white rot fungus native to the Yucatan Peninsula in Mexico, it produces laccases and decolorizes textile dyes. The efficient application of laccases in effluent treatments requires enzymes that can resist the processing conditions which usually contain acids, alkalis, salts, metals and organic solvents (Laing, 1991).

This study reports the purification and characterization of a laccase obtained from the white rot fungus *T. hirsuta* Bm-2 and its use in dye and effluent decolorization.

MATERIALS AND METHODS

Fungal strain and culture conditions

A fungal strain, *T. hirsuta* Bm-2 collected from decayed wood in Yucatan, Mexico was used in this work. The growth medium for propagation consisted of 20 g malt extract and 20 g agar per liter (pH 6.0). Plates were incubated for 4 days at 35°C.

Laccase production

The liquid medium to obtain inoculum was (per liter): 10 g glucose, 10 g malt extract, 2 g peptone, 2 g yeast extract, 2 g KH_2PO_4, 2 g $MgSO_4.7H_2O$ and 1 mg thiamine-HCl. 250 ml Erlenmeyer flasks containing 30 ml of medium were inoculated with two 1-cm^2 agar pieces from an actively growing fungus on malt extract plate (Bm-2). Flasks were incubated at 35°C and shaken at 150 rpm. After 4 days, the culture was homogenized using a sterilized blender. The liquid media for laccase production were a basal medium adjusted to pH 6.0 and induced medium containing 2% (wt/vol) wheat bran flakes in 60 mM phosphate buffer (pH 6.0). Two milliliters of mycelia suspension was inoculated to 100 ml medium and the culture was grown at 35°C for 10 days. Aliquots of the growth medium were withdrawn each 24 h, filtered and centrifuged to remove the mycelium.

Assay for laccase activity

Laccase activity in cell free filtrates was measured at 40°C using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS). The assay mixture contained 1 M sodium acetate buffer (pH 5.0) and 0.5 mM ABTS in a total volume of 1 ml. The oxidation of ABTS was measured by increase in absorbance at 420 nm and laccase activity was calculated from the molar extinction coefficient (ε) of 36,000 M^{-1} cm^{-1}. One unit of laccase activity is the amount of enzyme that oxidizes 1 µmol of ABTS min^{-1}ml.

Purification procedure

The supernatant from induced culture (7 L) was filtered, frozen overnight at -20°C and centrifuged at 15,000 rpm. Free cell culture was concentrated 100 times by liophylization. Laccase was precipitated by ammonium sulphate (60% saturation) and the precipitate was collected by centrifugation. The precipitate was dissolved in approximately 10 ml 0.1 M acetate buffer (pH 5.0). An extensive buffer exchange was performed using 20 mM Tris-HCl (pH 7.3) in dialysis membrane of 12 kDa (cut-off). The concentrate supernatant (2.8 mg protein) was loaded onto an ion-exchange (Q-Sepharose) column fast flow (45 x1.5 cm) equilibrated with the same buffer. The retained protein was eluted in stages using an NaCl gradient (0 to 0.5 M) at a flow rate of 3 ml/min. Fractions with laccase activity were pooled, concentrated, dialyzed and loaded onto a Sephacryl-200™ fast flow column (1 x 1.5 cm) which was equilibrated with 50 mM acetate buffer (pH5.0) containing 0.15 M Na Cl at a flow rate of 0.5 ml/min. The laccase peak was pooled, concentrated and dialyzed against 50 mM acetate buffer (pH 5.0). Fractions containing laccase activity were collected and concentrated. Protein estimation was done by Bradford (1976) method, with bovine albumin as standard.

Electrophoresis and zimograms

SDS-PAGE was performed according to the method of Laemli (1970) using a 12% polyacrilamide gel and Tris/glycine buffer 5X. The samples were treated with 10% SDS and 14.4 mM mercaptoethanol and boiled at 100°C for 10 min. Electrophoresis was carried out 2.5 h at 100 V using high molecular-mass standards (sigma). Proteins were visualized by silver staining (Galliano et al., 1991). Zymograms were obtained by renaturing the gels in buffer 10 mM Tris-HCl (pH 7.0) for 2 h. The oxidation zones produced by laccases were revealed with a solution of 2.5 mM ABTS in 0.1 M acetate buffer (pH 5.0).

Optimum temperature, pH and stability

The temperature profile of the laccase was studied by measuring the activity in a range of 30 to 75°C. LacI solution was incubated in 1 M sodium acetate buffer (pH 5.0) at different temperatures and the activity was determined with ABTS as substrate.

The pH-dependence of the laccase activity was carried out at 25°C using 0.05 mM ABTS in different buffer solutions, 1 M: glycine/HCl buffer (pH 3.0 to 3.5), sodium citrate buffer (pH 4.0 to 5.0) and sodium phosphate buffer (pH 6.0 to 6.5).

The thermal stability of LacI was determined by following the oxidation of ABTS of 5 mM ABTS at optimum pH and temperature after pre-incubation of laccase for 60 min at 40, 45, 50, 55 and 60°C. For pH stability, LacI extract was pre-incubated at room temperature in different buffers at pH 3.0 to 6.5. Aliquots were removed after one hour of incubation and assayed at optimum pH and temperature.

Stability in organic solvents

The stability of laccase against organic solvents was assessed. Laccase was incubated with ethanol and acetonitrile 20% at room temperature for 24 h. Residual activity was determined using ABTS assay at optimum pH and temperature.

Substrate specificity

Spectrophotometric measurement of substrate oxidation by LacI

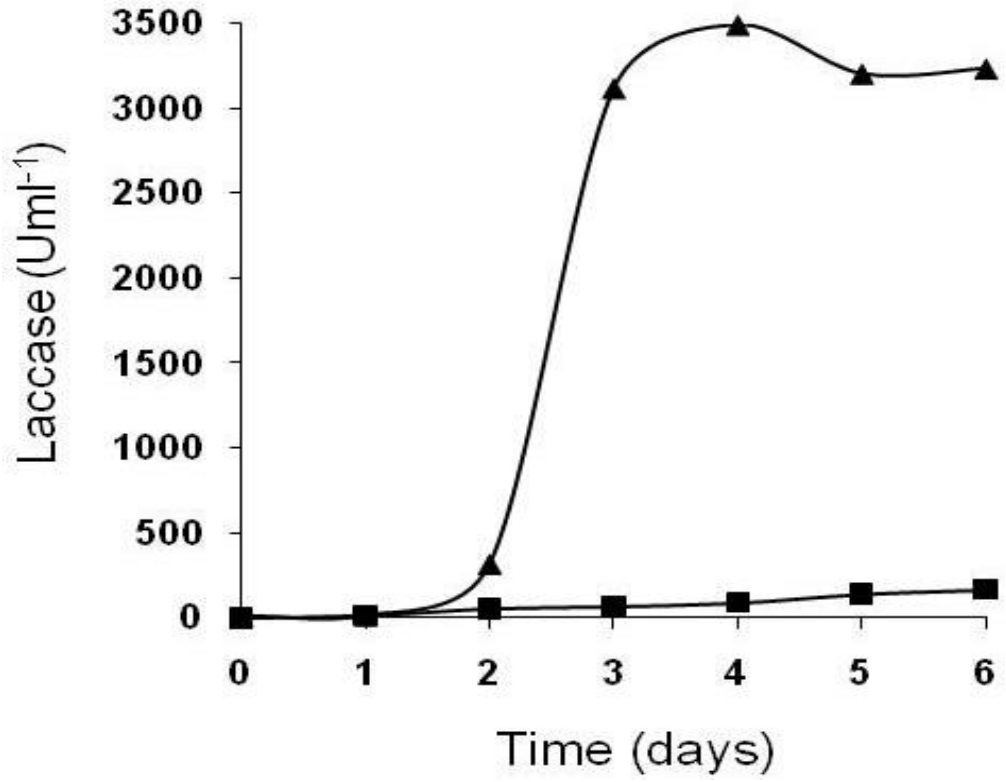

Figure 1. Time course of laccase production by *T. hirsuta* Bm-2 during growth in basal medium (■) and wheat bran (▲)

was carried out in a 1 ml reaction mixture containing the test substrates. Activity against ABTS and 2,6-dimethoxyphenol (DMP) was assayed at concentrations between 0.5 and 2.0 mM. All reactions were carried out at 45°C and pH 4.5. The rate of substrate oxidation was determined measuring the absorbance increase, at 420 and 470 nm for ABTS and DMP, respectively. The molar extinction coefficient (ε) were 36,000 (ABTS), 35,645 (DMP). Apparent kinetic constants (*Km, Vmax*) were calculated using Lineweaver-Burk plots.

Effect of ions and inhibitors

The activity of laccase was tested in the presence of several metal ions including Cu^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+} and Cd^{2+}. The effect of potential inhibitors of the enzyme activity was determined in the presence of the following substances: cysteine, SDS, sodium azide, and EDTA were evaluated at two concentrations (1 and 10 mM). The residual activity was determined using ABTS assay.

Decolorization of effluent and textile dye

Decolorization of textile dye and real effluent was investigated by the crude and purified enzyme (LacI). The reaction mixture contained 1 M sodium citrate buffer at pH 4.5, acid blue (50 mg L^{-1}) and laccase (193 U/mg protein). The reaction was initiated with enzyme addition and incubated at 45°C. Samples were withdrawn at different intervals and subsequently analyzed. Residual color was detected at 600 nm using a spectrophotometer. Reaction mixtures containing dyes or effluent without enzyme were used as control. Decolorization was calculated with reference to the zero hour reading.

Statistical analysis

The data are the average of the results of three replicates with a standard error of less than 5%.

RESULTS

Production and purification of the lacasse from *T. hirsuta* Bm-2

The production of extracellular laccase was studied in *T. hirsuta* in a saline medium and supplemented with wheat bran. The enzyme was produced in both media, however, in the medium with wheat bran, laccase production increased significantly at day 3 and maximum activity was obtained at day 4 of culture (2496 U/ml), representing an activity 14 times greater in comparison with the saline medium (Figure 1). Manganese peroxidase and lignin peroxidase activity were not detected in the extracts.

The laccase secreted by *T. hirsuta* Bm-2 was purified by two step chromatography (Figure 2). After anion exchange chromatography with Q-Sepharose, part of the

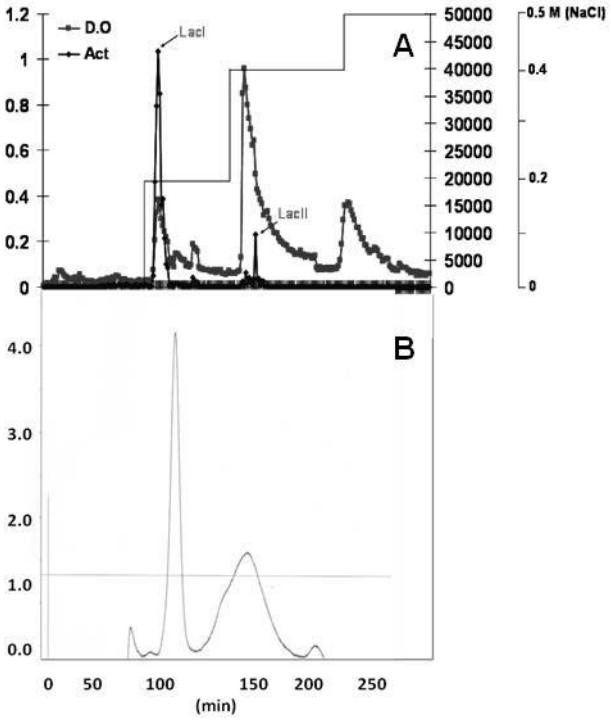

Figure 2. Purification of laccases from *T. hirsuta* secreted in medium with wheat bran: (A) Q-sepharosa fast flow. Absorbance at 280 nm (O), laccase activity (•), NaCl step gradient is indicated, (B) Sephacryl S-200.

Table 1. Purification steps for laccase from *T. hirsuta* Bm-2.

Steps	Total protein (mg)	Total activity (U)	Specific activity. (U/mg)	Purification fold	Yield (%)
Crude extract	553.8	10, 515, 100	18, 987	1.00	100
Ammonium sulphate	52.7	4, 141, 126	78, 626	4.10	39.38
Q-Sepharose					
LacI	13	2, 502, 774	193, 713	10.20	24
LacII	9.2	207, 774	22, 633	1.20	2
Sephacryl S-200					
LacI	0.243	58, 404	240, 345	14.05	11.42

protein was eliminated and the dark brown pigment present in the crude extract was also eliminated, as has been reported in other studies (Cordi et al., 2007; Minussi et al., 2007). The protein peaks in fractions 76-88 and 137-154 showed laccase activity and were denoted as LacI and Lac II. The main laccase was LacI (Figure 2A) with an activity of 44,860 U/ml, while LacII was 9,860 U/ml. During gel filtration chromatography with Sephacryl-200, a protein peak with laccase activity was resolved and separated from other contaminating proteins. Table 1 summarize the purification steps. The main laccase was purified 14 times with a yield of 11%. The crude extract and chromatographic fractions showed an intense protein band during gel SDS-PAGE running, indicating positive oxidation of ABTS in the zimogram (Figure 3). The laccase appears to be a monomeric protein with an apparent molecular mass of 65 kDa.

Characterizaton of the purified laccase

The effect of varying pH and temperature on laccase activity was investigated (Figure 4). Optimum pH for LacI was from 4 to 4.5 using ABTS as substrate. Lac I was

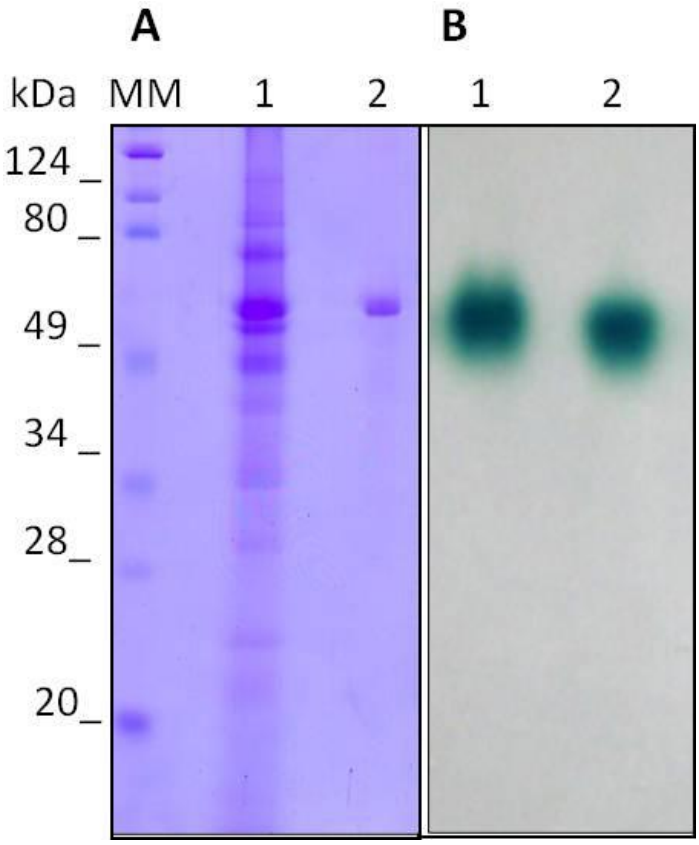

Figure 3. SDS-PAGE gel. Estimation of molecular weight (A) and zymogram of laccase from *T. hirsuta* Bm-2. Line 1: crude extract, line 2: fraction from Sephacryl S-200. MM: molecular weight markers.

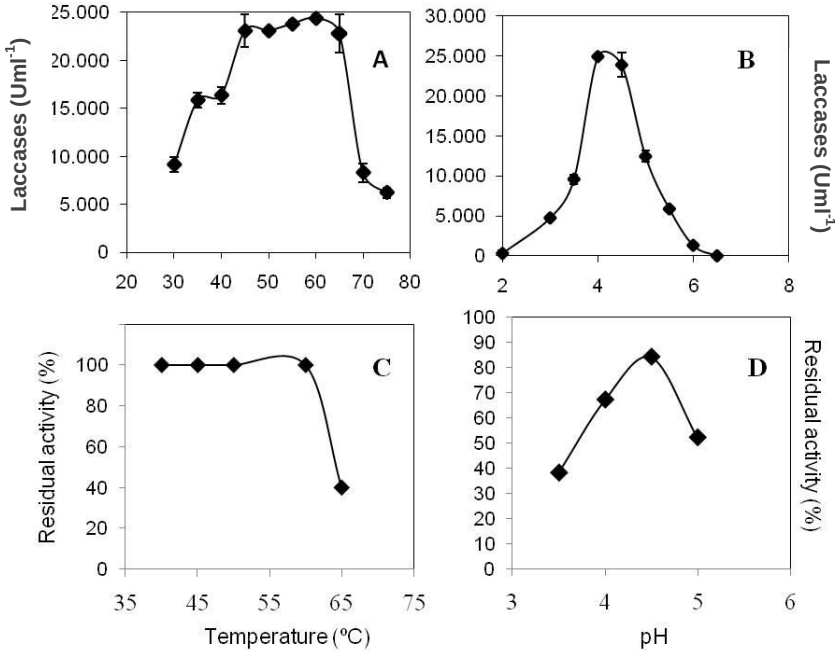

Figure 4. Effect of temperature and pH activity (A and B) and temperature and pH stability (C and D) of the purified laccase (LacI) of *T. hirsuta* Bm-2.

Table 2. Effect of different compounds on laccase from *T. hirsuta* Bm-2.

Compound	Relative activity	
	(1 mM)	(10 mM)
Control	100	100
Mg^{2+}	186	73
Cu^{2+}	92	100
Ca^{2+}	100	54
Mn^{2+}	40	20
Cd^{2+}	87	89
Cisteine	0	0
SDS	0	0
Sodium Azide	22	2
EDTA	73	0
Ethanol*		100
Acetonitrile*		100

Activity was determined at 24 h. *Concentration of organic solvents was 20%

Table 3. Kinetic constants of purified laccase LacI from *T. hirsuta* Bm-2.

Substrate	Wavelength	ε (mM^{-1} cm^{-1})	*Km* (µM)	*Vmax* (µM)
ABTS	420	36000	68	14
2,6-Dimethoxyphenol	467	35645	164	4.65

completely stable at pH 4 to 4.5 for an hour. The optimum temperature was within a wide range (40 to 60°C). Temperature stability was 100% during an incubation period of one hour at a temperature range of 40 to 60°C and retained 50% of activity at 65°C.

The effect of inhibitory compounds on laccase activity was determined (Table 2). Metals such as Mg^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+} and Cd^{2+} were added at concentrations of 1 and 10 mM using ABTS as substrate. With the exception of Mn^{2+} which proved to be a strong inhibitor of the enzyme, the metallic ions did not significantly affect activity and the addition of the ion Mg^{2+} 1 mM activated the laccase (186%). The sensitivity of the laccase to typical inhibitors of the enzyme was also evaluated. The purified laccase was strongly inhibited by sodium azide, L-cisteine and SDS. One interesting finding was that laccase from *T. hirsuta* was very resistant to 20% ethanol and acetonitrile (Table 2).

Kinetic constants of the enzyme were determined for ABTS and DMF substrates (Table 3). Reaction time curves showed that the enzyme presented classic Michaelis-Menten kinetics. The values of apparent *Km* and *Vmax* were 68 and 164 µM for ABTS and DMF, respectively. These results showed that the enzyme has a higher affinity towards ABTS than DMF.

In this study, the ability of crude and purified enzyme to decolorize a synthetic dye and a textile effluent was examined (Figure 5). With crude extract, acid blue (indigo carmine) was removed completely after an incubation of 24 h. Decolorization with the purified enzyme was similar to that obtained with the crude enzyme. Also, the results of this work showed that the crude and purified laccase was able to reduce the color of a textile effluent by 42 and 37% respectively, indicating that these enzymes are directly involved in the decolorization process.

DISCUSSION

The production of laccases from fungi has been shown to depend on the composition of the medium and culture conditions; it is particularly affected by the presence of lignin or phenolic and aromatic monomers which induce the syntheses of these enzymes (El-Shora et al., 2008). The addition of natural substrates with lignin, such as corn-cob, sawdust, wheat straw and bagasse increased the production of extracellular lacasse in *Pleurotus sajor caju* MTCC 141 at 10 days of culture (Sahay et al., 2008). *T. hirsuta* Bm-2 produced a high level of laccase activity in a relatively short incubation period in comparison with other fungi, such as *Funallia trogii* (Patrick et al., 2009) and *Cerrena unicolor* (Kim et al., 2002) which produce maximum laccase activity at 15 and 12 days of culture, respectively.

Two laccases secreted by *T. hirsuta* were partially purified. The production of laccase isoforms from white

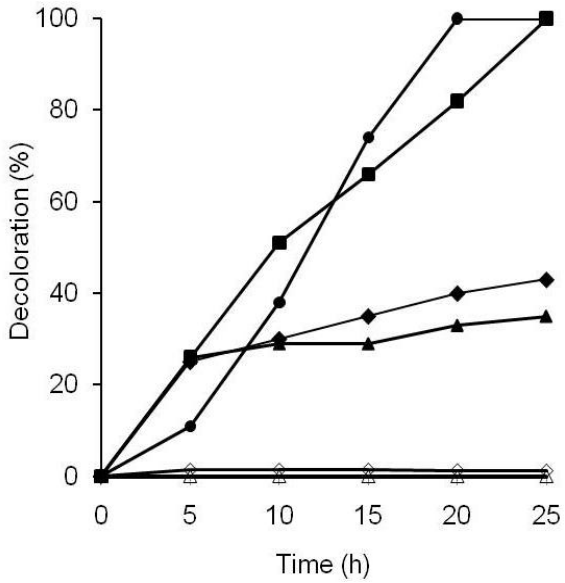

Figure 5. Decolorization of acid blue by crude extract (■), LacI (●), control without enzyme (Δ). Decolorization of a textile effluent by crude extract (♦), LacI (▲) and control without enzyme (◊).

rot fungi has been widely reported (Palmieri et al., 2003; Dantan-González et al., 2008) and several laccase genes have been described in *Pleurotus sajor caju* (Soden and Dobson, 2001). Although, numerous isoforms can be variants of the same gene due to postranscriptional modifications (Manzur et al., 1998), the major laccase (LacI) appeared to be a monomeric protein similar to other fungal laccases with a molecular mass apparent of 65 kDa. Shleev et al. (2004) reported the molecular mass of laccases from *Tabebuia ochracea, Coriolopsis fulvocinerea, Cerrena máxima* and *Trametes hirsuta* within a range of 64 to 70 kDa. In contrast with some basidiomycetes such as *T. versicolor* and *Fomitella fraxinea*, the molecular weight of the laccases ranged from 97 to 80 kDa (Moon-jeon et al., 2005, Park and Park, 2008). Variation in laccase size has been associated with the glycosylation degree of the protein.

The optimum pH of the laccase from *T. hirsuta* Bm-2 was 4.5 with ABTS as a susbstrate. Studies with laccases from *T. hirsuta, Trichoderma harzianum WL1*, *Pleurotus sajor-caju* MTCC141 and *T. versicolor* have shown a pH of 4.5 to be optimal for laccases (Sadashivan et al., 2008; Sahay et al., 2008). However, in *Trametes trogii* the highest oxidation rate was obtained at pH 2 for ABTS (Zouari-Mechichi et al., 2006). Although pH optima for laccases can vary depending on the substrate used and on its redox potential (Soden et al., 2001). In the range of pH values evaluated, the enzyme was stable at pH 4.5. Stability of the laccase in this study was highest at its optimum pH. Fungal laccases are usually stable at acidic pH, although pH stability varies considerably depending on the source of the organism (Baldrian, 2006).

Laccase of this study showed optimum temperature and stability over a broad temperature range (40 and 60°C). The temperature stability varies considerably. In general, laccases are stable in a range of 25 to 50°C, however rapidly lose activity at temperatures above 50°C (Ko et al., 2001; Sadhasivam et al., 2008, Moon-Jeon et al., 2005). For industrial applications of laccases, thermal stability is an important desirable property. Although, some fungal laccases are thermostable, most of these enzymes lose activity at temperatures above 50°C (Cambria et al., 2000; Das et al., 2001). The thermal stability at high temperatures of the laccase from *T. hirsuta* Bm-2 was a considerable advantage in the purification process which was carried out at room temperature, since most purification studies are conducted under refrigerated conditions. The thermostability of LacI could be a very useful characteristic in industrial applications.

The application of laccases in effluent treatment requires enzymes resistant to metallic ions and inhibitors, therefore it was important to find some resistance of laccases from *T. hirsuta* to metallic ions. This is not a common behavior in fungal laccases which are usually sensitive to various cations (Robles et al., 2002). De Souza et al. (2003) reported that purified laccase from *Pleurotus pulmonaris* was resistant or partially resistant to Fe^{2+}, Ni^{2+}, Mn^{2+} and that copper stimulated enzyme activity. Similar to other laccases, LacI from *T. hirsuta* was strongly affected by some compounds. Sodium azide

constitutes the most effective inhibitor of many oxidative enzymatic reactions and links to type 2 and 3 copper sites, impeding the transfer of electrons during catalysis of the enzyme (Ryan et al., 2003). L-cisteine is also a classic inhibitor of phenoloxidase activity. Although, SDS is considered as a denaturalizing agent of proteins, its effect on laccase activity has been quite variable. The laccase of *T. hirsuta* was mildly inhibited by EDTA (10 mM). Fungal laccases are quite frequently inhibited by EDTA 1.0 mM (Younes et al., 2007). However, a few exceptions such as laccases of *Marasmius quercoplhilus* (Farnet et al., 2002) and *Phellinus ribis* (Min et al., 2001) have been described, which are only inhibited by high concentrations of EDTA.

Resistance to water miscible solvents is an important property in laccases considering that many transformations must be carried out at high concentrations of organic solvents. The *T. hirsuta* laccase maintained up to 100% activity in the presence of 20% of the ethanol and acetonitrile for 24 h. Laccases generally show high sensibility to unfolding in the presence of these compounds (Al-Adhami et al., 2002). To improve this property, molecular evolution studies were carried out to improve laccase activity in the presence of 20% acetonitrile and 30% ethanol (Alcalde et al., 2005). Our enzyme has the possibility to work in the presence of organic solvents. This is an important feature because this laccase could be a good candidate for use in the treatment of textile effluents.

The *Km* value for the laccase purified from *T. hirsuta* showed more affinity to ABTS than 2,6-dimethoxyphenol. This difference may be correlated to their structures. In general, laccases are known to posses very wide range substrate affinities, however the most of the laccases characterized show greater affinity for ABTS (Baldrian, 2006).

Fungal laccases as well as laccase-mediator systems are efficient in dye decolorization (Abadulla et al., 2000; Zille et al., 2005). The laccase of *T. hirsuta* was also responsible for the decolorization of the blue acid dye. The result concur with reports for *T. versicolor* laccases which were able to decolorize acid blue (97.5%) without the addition of a redox mediator (Stoilova et al., 2010), while the purified laccase of *Cerrena unicolor* and *T. versicolor* improved the decolorization speed for textile dyes when acetosyringone was added as mediator (Cho et al., 2006). This discrepancy regarding the use of a mediator or not can be attributed to the difference in redox potential of the enzyme which may vary depending on the source of laccases (Li et al., 1999).

The reduced efficiency in effluent decolorization with respect to acid blue by laccase from *T. hirsuta*, was attributed to the complexity and high concentration of the compounds present in the effluent. Cordi et al. (2007) found that after treating a Kraft effluent with the immobilized laccase of *T. versicolor*, the phenolic content min. Diaz et al. (2010) reveled that laccase preparation was reduced by only 10% after a treatment period of 180 from *Coriolopsis rigida* reduced 42% soluble aromatics including free phenols from solid waste from olive oil production. There have also been reports on *in vivo* decolorization through the cultivation of fungi in effluents. Salony and Bisaria (2007) reported that the decolorization efficiency in an olive mill black liquor effluent, during the culture of the fungi *Cyatus bulleri,* improved phenol elimination from 30 to 90% when the dilution was increased from 1:10 to 1:50, suggesting that the laccase may be inhibited by toxic substances present in the effluent.

The results of this study show that the *T. hirsuta* laccase has a number of properties similar to those of other fungal laccases; but more importantly, it also highlights important characteristics, such as its high activity and resistance to organic solvents as well as its stability in a wide range of temperatures, allowing it to be included in the small group of thermostable laccases. Moreover, LacI demonstrated a high decolorization capacity in a synthetic dye and textile effluent, making this enzyme of particular interest in further research to optimize the biotreatments of colored effluents from the textile and dye industries.

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