Chapter 15

Purification and Partial Characterization of a Thermostable Laccase from *Pycnoporus sanguineus* CS-2 with Ability to Oxidize High Redox Potential Substrates and Recalcitrant Dyes

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Additional information is available at the end of the chapter

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1. Introduction

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are enzymes that catalyze the oxidation of phenolic compounds and aromatic amines with the simultaneous reduction of molecular oxygen to water [1]. They are widely distributed in many plants and fungi, some insects and bacteria, being particularly abundant in white-rot basidiomycetes [2]. Typical fungal laccases are described as glycosylated multicopper proteins, which are produced as extracellular monomeric forms of around 60-80 kDa, containing four copper atoms and 15-20% carbohydrates. Operatively, they are moderately thermotolerant, showing optima activity at 50-55 °C, and under acidic conditions (pH 3-5); although their maxima stability occurs in the alkaline zone (pH 8-9) [3]. Their copper atoms are distributed in three different sites bringing unique spectroscopic properties: The type 1 copper (CuT1) atom, is responsible of the intense blue color of enzymes by light absorption around 610 nm; The type 2 copper (CuT2) atom exhibits a weak absorption in the visible region; and the two type 3 copper (CuT3) atoms are present as a binuclear center, which has an absorption maximum about 330 nm. Moreover, CuT2 and CuT3 copper atoms are structural and functionally arranged as a trinuclear cluster. The four copper atoms form part of the active site of enzyme contributing directly to reaction. CuT1 is involved in the initial electron subtraction from reducer substrates, while trinuclear

CuT2 and CuT3 cluster is responsible of the electron transference, from CuT1 to diatomic oxygen [4].

According to their redox potential, most of blue laccases belong to class II (-500 to -600 mV) or class III (-700 to -800 mV) laccases [5]. This is a disadvantage when compared to the ability of lignin peroxidase (LiP) and manganese peroxidase (MnP) to attack compounds with higher redox potential, including non-phenolic lignin units. To overcome this limitation laccases have evolutively developed a synergistic catalytic strategy, which combines a flexible ability to recognize a great variety of chemical compounds, with an extended capability to act at the distance through the activation of diffusible low molecular substances which serve as redox mediators. From a biological stand point this strategy let laccases to become one of the most versatile enzymes in nature, adaptable to multiple functions in plants, insects, fungi and bacteria. Another interesting possibility arises from the properties of atypical "yellow" and "white" laccases, which have shown the ability to catalyze the direct oxidation of high redox potential non-phenolic lignin model substrates or polyaromatic hydrocarbons [6, 7]. It has been proposed that the improved redox capabilities of these laccases come up either, by substituting some copper atoms for zinc, iron, or manganese in the metal clusters or by a change of the redox state of the CuT1(due to the interaction with a lignin-derived ligand) at the active site of, otherwise normal laccase protein structures. So, evolution and prevalence of laccases as a part of the lignin modifying enzyme (LME) system in white-rot basidiomycetes could also be the result of a "biochemical spring-up" mechanism acting under a short term ecophysiological selective pressure.

Whether directly or by mediation, laccases are able to oxidize a broad range of natural or xenobiotic compounds, including: mono, poly or methoxy- amine- and chloro-substituted phenols as well as aromatic heterocyclic and inorganic/organometallic substances; some of them recognized among the most recalcitrant industrial pollutants, for example; polycyclic aromatic hydrocarbons (PAH), pentachlorophenols (PCP), polychlorinated biphenyls (PCB), 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), trinitrotoluene (TNT), and many azo, triarylmethane, anthraquinonic, indigoid and heterocyclic textile dyes [8,9]. Therefore, laccases are considered enzymes with a great potential for the development of environmental and industrial applications. Current and potential laccase applications include biobleaching of pulp and bioremediation of pulp and paper industries, bioremediation of olive mill wastewater, bioremediation of effluents of the textile and dye manufacturing industries, biocatalytic synthesis of antibiotics and novel polymeric materials, development of biosensors, clarification and stabilization of beer, juices and wines, and panification [1, 10, 11]. Some laccase formulations have already reached a commercial significance, but general thought is that their biotechnological applications and performances could be greatly improved or expanded with the development and finding of new enzyme variants with desirable functional properties, such as higher redox potential, optimum activity at neutral or alkaline pH and thermal stability [12, 13, 14]. It has been proposed that these new laccases could be obtained by protein engineering or through the exploration of the natural biodiversity. The importance of prospective studies in natural biodiversity applying an ecophysiological approach is illustrated by reports about isolation of new thermostable laccases from fungi, either from thermophilic

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compost [15] or tropical environments [13,16], or by the finding of novel laccases with improved ability to oxidize substrates with a higher than normal redox potential culturing under solid phase conditions [6, 7,17]. Northeast Mexico shelters a high diversity of white-rot basidiomycetes as a result of its particular combination of physiography and climate, including species associated to pine, oak and mixed forests, sub-mountain and semi-desert scrublands, and grass-land. In this work we first present information on the isolation, identification and selection of a northeast Mexico native strain of *Pycnoporus sanguineus* CS2, as a potential producer of thermostable laccases. Results on the purification and partial characterization of its laccase are then exposed, stressing on its thermal stability and ability to attack high redox potential substrates and recalcitrant dyes without the participation of redox mediators.

2. Materials and methods

2.1. Chemicals

All chemicals used as buffers, enzyme substrates, culture media ingredients and electrophoresis reagents, were reactive grade and commercially available through local distributors of Difco, Sigma-Aldrich and Fluka, or BioRad products: PDB (potato dextrose broth), bacteriological agar, yeast extract, malt extract, peptone and dextrose, were from Difco. Acrylamide, bis-acrylamide, TEMED (N,N,N′,N′- tetramethylethylenediamine), 2-mercaptoethanol, SDS (sodium dodecyl sulfate), trizma-base, glycine, Coomassie blue, and low range markers kit, were from Bio Rad. Enzyme substrates and dyes: 2,6-dimethoxyphenol (2,6-DMP); *o*-dianisidine (3,3′-dimethoxybenzidine); ABTS (2,2′–azino-bis(3-ethylbenzthiazolin-6-sulphonic acid); syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine); DMAB (3-dimethylaminobenzoic acid), MBTH (3-methyl-2-benzothiazolinone hydrazone); Methyl Red (Acid Red 2; CI 13020); Reactive Black 5 (RB 5; CI 20505), were from Sigma, Fluka or Aldrich. Chromatographic matrices; DEAE-Sepharose and Q-Sepharose from Sigma-Aldrich, and Biogel P-100 from BioRad. All other chemicals, including solvents, inorganic salts, acids and bases, were from Reactivos Químicos Monterrey, S.A. or CTR-Scientific S.A. de C.V. Solutions and culture media were prepared with bidistilled water from Laboratorios Monterrey, S. A.

2.2. Isolation and identification of fungal strain

The *Pycnoporus* strain used in our experiments was isolated from fruit bodies developing on decayed logs that were gathered in a man-disturbed sub-mountain scrubland around Monterrey, N.L. (Northeast México). Mycelia cultures were obtained by standard mycological techniques, according to the procedure previously described [18]. Briefly, small flesh sections were aseptically removed from inside the carpophores, and transferred to YMGA (0.4% Yeast extract, 1.0% Malt extract, 0.4% Glucose, 1.5% agar) plates, supplemented with 10% Tartaric Acid and 0.004% Benomyl. Plates were incubated at 28 °C, and those with extensive mycelia growth were analyzed under the microscope to confirm a successful isolation. Stock cultures were maintained by periodic transfers every two or three months on YMGA plates and kept refrigerated at 4 °C. Carpophore morphologic features, measurements and photographs were

registered previous to dissections for microscopic examination, and identification was done by following the taxonomical keys in reference [19], and in [20] for genera of polypores and the most common macromycetes from Mexico, respectively.

2.3. Enzyme and protein assays

Laccase activity was determined by triplicate at 25 °C in 3 ml cuvettes, monitoring the increase in absorbance at A_{468} (ε=49,600 $M^{-1}cm^{-1}$), using a Shimadzu UV-VIS mini 1240 spectrophotometer and 2,6-DMP as substrate. The assay mixture contained 0.01ml enzymatic extract, 0.1 ml of 60 mM 2.6-DMP in 2.89 ml of 200 mM citrate-phosphate buffer at pH 4.0 [21]. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of 2, 6-DMP per minute at 25 °C. In some cases, it was necessary to assess the presence of lignin peroxidase (LiP) and manganese peroxidase (MnP). LiP activity was estimated by the H_2O_2-dependant veratryl alcohol oxidation to veratraldehyde as in reference [22] MnP activity was measured by the formation of Mn^{3+}-tartrate complex during the oxidation of $MnSO_4$ in tartrate buffer as in [23]. The protein concentration was estimated by the Bradford assay (Protein Assay Bradford of BioRad) with bovine serum albumin as standard.

2.4. Strain selection and enzyme production

Isolated *Pycnoporus* strain was selected as a potential source of thermostable laccases in a preliminary screening with crude enzyme preparations. 250 ml Erlenmeyer flask, containing 50 ml of natural LME inducers containing Bran Flakes (BF) media (2% Bran Flakes® in 60 mM potassium phosphate pH 6.0) [24], were inoculated with three 0.5 cm diameter cylinders of mycelia taken from the border of a YMGA growing colony and incubated at 28 °C under agitation at 150 rpm. Aliquots (200 μl) were removed from cultures and the extracellular fluid was separated by centrifugation at 14 K (Eppendorf 5415 C). Enzyme activity in supernatants was determined with 2 mM 2, 6-DMP final concentration, as described above, after sample incubation at 60 °C during different times in a four hour period. This phase of study included four different *Pycnoporus* sp. (CS 2, CS 20, CS 43 and LE 90) strains from the native basidiomycete collection of our laboratory. Among them, *Pycnoporus sanguineus* CS 2 was selected on the basis of its ability to produce a thermostable 2,6-DMP oxidizing activity, and for showing apparently a single band of activity when incubated with 2,6-DMP and SGZ in a parallel native PAGE analysis of crude supernatants.

Enzyme production was evaluated in submerged liquid cultures on the natural containing laccase-inductors BF or a modified Kirk medium (MK) [25], with the following composition: 10 g l^{-1} dextrose, 1.0 g l^{-1} yeast extract, 5.0 g l^{-1} peptone, 2.0 g l^{-1} ammonium tartrate, 1.0 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4$, 0.5 g l^{-1} KCl, and 1.0 ml of 100 X trace element solution (0.5 g EDTA, 0.2 g $FeSO_4$, 0.01 g $ZnSO_4$, 0.003 g $MnCl_2$, 0.03 g H_3BO_4, 0.02 g $CoCl_2$, 0.001 g $CuCl_2$, and 0.003 g $NaMoO_4$ in 100 ml); amended with 350 μM $CuSO_4$ and 3% ethanol [26]. Cultures were performed at 28 °C and 150 rpm for 14 days. 50 μL aliquots were taken every two days to determine the laccase activity.

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2.5. Laccase purification

All the procedures were performed at 4 °C, unless otherwise stated. Extracellular liquid from 14 day-old submerged cultures was separated from mycelium by filtration through a cotton-polyester 50:50% cloth. Then, water-soluble polysaccharides were removed from sample solution by freezing (- 20 °C for 24 h), thawing and filtration (Whatman # 1). Culture filtrate was concentrated to approximately 200 ml by 10 kDa ultrafiltration (Millipore prep/scale TFF cartridge). The obtained fluid was further reduced to 20 ml by using a stirred ultrafiltration system equipped with an YM10 membrane (Amicon, Millipore). The reddish-brown enzyme concentrate was equilibrated by diafiltration with 20 mM potassium phosphate, pH 6.0, and applied to a pre-equilibrated anion-exchange DEAE-Sepharose column (2.5 × 17 cm). Once on the column, unadsorbed protein and most of the pigment were removed by washing with two volumes of equilibrium buffer. Retained proteins were eluted with a linear gradient of potassium phosphate pH 6.0 from 20 to 300 mM, and the eluted fractions were assayed for laccase activity and the A_{280} nm monitored. Fractions with laccase activity were pooled, concentrated, equilibrated by diafiltration with 100 mM potassium phosphate, and applied on a pre-equilibrated Biogel P-100 column (2.6 x 65 cm). The loaded proteins were eluted with the same buffer. Active fractions were pooled, concentrated and diafiltrated against 20 mM potassium phosphate buffer pH 6.0. Enzyme was further purified by anion-exchange on a pre-equilibrated Q-Sepharose column (2.5 x 17 cm). Once set the sample, active fractions were eluted with a lineal gradient of potassium phosphate from 20 to 300 mM. These fractions were pooled, concentrated and diafiltrated against water, and stored at - 20 °C.

2.6. Electrophoresis analysis

Protein purity and molecular mass were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as in reference [27] with 4% stacking gel and 12% resolving gel. Protein bands were stained with Coomassie brilliant blue and the molecular mass (M_r) of purified laccase was determined by calculating the relative mobility of standard protein markers: *phosphorylase b* 97.4 kDa; *serum albumin* 66.2 kDa; *ovalbumin* 45 kDa; *carbonic anhydrase* 31 kDa; *lysozyme* 14.4 kDa; *aprotinin* 6.5 kDa (SDS-PAGE molecular weight standards low range, BioRad). Native PAGE was carried out as described at reference [28]. Activity staining of laccase was performed by incubating with 2, 6-DMP or the pair MBTH + DMAB in 200 mM sodium acetate buffer, pH 4.5. For identification and comparing proposes, the corresponding laccase band was removed from a parallel gel and submitted to the Proteomic Unit of IBT-UNAM at Cuernavaca, Morelos for aminoacid sequencing; resulting in the sequencing of six internal peptides.

2.7. UV-Vis absorbance spectra

As a part of the characterization of the physicochemical properties of the laccase, its absorbance spectrum from 200 to 800 nm was obtained in a UV-Vis Shimadzu-Mini 1240 spectrophotometer. The assay was performed with 25 μM of protein diluted in 1 ml of bidistilled water.

2.8. Effect of pH on enzymatic activity

Optimum pH of activity was determined in McIlvine buffer (consisting of a combination of 100 mM citrate/50 mM potassium phosphate) adjusted in a range from 3.0 to 7.0. Activity determination was made according to described method with 2,6-DMP using 0.2 M citrate phosphate buffer, at pH 4.0.

2.9. Effect of temperature on enzyme activity and stability

The effect of temperature on reaction rate was determined using 2, 6-DMP in 0.2 M citrate/phosphate buffer, at pH 4.0. The temperature of reaction mixture was adjusted to indicate value and then the reaction was started by the addition of enzyme. The assays were done by triplicate and data in graphics appear as relative activity as a function of temperature, considering as 100% the average of maxima obtained. The activation energy of the system was calculated by the Arrhenius model, according to the expression: Log k = [-E_a / 2.303R (1/T)] + Log A, where: k is the rate constant (it depends of temperature); A is preexponential factor or frecuency factor. E_a the activation energy (expressed in J/mol); R is the gas universal constant (8.314 J K^{-1} mol^{-1}), and T the absolute temperature (°K). In the thermostability assays, the enzyme was pre-incubated at 50, 60 and 70 °C for the indicated periods of time and activity was measured at 25 °C on 2, 6-DMP in 0.2 M citrate/phosphate buffer, at pH 4. The assays were carried out by triplicate and data are expressed as percent of remaining activity as a function of incubation time; taking as 100% the average value of activity at time zero for each temperature. Inactivation process was adjusted to an exponential decay model, from which the constants of heath inactivation (k) and the half-life times were calculated according to the expression: ln (N_o/N) = kt, where; N_o is the activity at the starting of incubation; N is remaining activity after a certain incubation time (t); k correspond to the first-order inactivation constant, and $t_{1/2}$ is the half-life time, calculated as ln 2/k = 0.693/k

2.10. Determination of kinetic parameters

Kinetic analysis was performed on some common substrates of laccase: 2, 6-DMP, ABTS, *o*-dianisidine and SGZ. Reaction mixtures were prepared in 0.2 M citrate/phosphate buffer at pH 4.0 and the change in optical density by minute was measured by triplicate at different substrate concentrations (0.05, 0.1, 0.5, 1.0, 5.0, 10.0 mM) or those indicated for each assay. The assays were performed at 468 nm for 2, 6-DMP (ε = 49,600 M^{-1} cm^{-1}), 436 nm for ABTS (29,400 $M^{-1}cm^{-1}$), 460 nm for *o*-dianisidine (11,000 M^{-1} cm^{-1}) and 525 nm for SGZ (ε= 65 000 M^{-1} cm^{-1}). The values of the Michaelis constant (K_m), maximum velocity (V_{max}), turnover number (K_{cat}) and specificity constant K_{cat}/K_m were estimated according to the Lineweaver-Burk method.

2.11. Decolorization assays

The decolorizing ability of laccase was evaluated with two recalcitrant dyes, the non-phenolic azo Methyl Red (MR), and the diazo reactive black 5 (RB 5). The reaction mixture consisted of 0.890 ml of 0.2 M citrate/phosphate buffer, pH 4.0, 0.1 ml of 250 μM MR or RB5 (final concentration 25 μM), and 0.01 ml of pure laccase (final concentration 5 U/ml). Assays were performed

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at 25 °C and reaction was initiated with the addition of enzyme. Decolorization was estimated by the decreasing of absorbance at 530 nm for MR or 597 nm for RB5. The results are expressed as the percent of remaining color as a function of incubation time according to the relationship: remaining color (%) = [(Abs_{final}/ $Abs_{initial}$)]*100, where: Abs_{final} correspond to the absorbance value at the indicated incubation times, and $Abs_{initial}$: is the initial (t = 0) absorbance value.

3. Results and discussion

3.1. Strain identification

In this study an autochthonous strain of *Pycnoporus* sp (CS 2) was selected as a potential source of thermostable laccases for its ability to produce a thermotolerant 2, 6-DMP oxidizing activity in preliminary assays with crude filtrates from submerged cultures. This basidiomycete was initially isolated from fruit bodies, growing on decayed logs in a man disturbed sub-mountain scrubland around Monterrey, N.L. México (Figure 1), and identified by its morphological and microscopic traits. According to their morphological and microscopic characteristics, the carpophores corresponded to the species *Pycnoporus sanguineus* (L.) Murrill, for their bright orange to orange-red, red or cinnabar-red shelf-like basidiomes, which are nearly round to elongated or fan-shaped in outline, have a dry surface, smooth or finely hairy, wrinkled or warty and attain 2-12 cm diameter and 0.2 to 0.5 cm thick. Their margins are thin and the under surfaces are covered by small pores (3-4 per mm), bright orange to orange red or red ranging from 0.5-1.5 mm long. Their white spores are smooth and oblong-elliptical in shape and range from 4.2 to 5.2 microns long by 2 to 3.5 microns width and the flesh is tough, red to yellowish red, staining black with KOH. On the bases of these features, we assigned the strain under study as *Pycnoporus sanguineus* CS 2.

3.2. Production and purification of laccase

Guzmán (2003), considers that *P. sanguineus* is a tropical variant of the temperate zone species *P. cinnabarinus*, adapted to man disturbed sites, where it is common in fallen logs and fences, always in sunny places [29]. As its closely related species, *P. cinnabarinus* and *P. coccineus*, *P. sanguineus* is recognized as an efficient lignin decomposer, in spite of its relatively simple LME system composed of laccases [5]. These features make *Pycnoporus* species an attractive group of white-rot basidiomycetes for the production and purification of unusual laccases [16, 30]. In this study, laccase production was carried out in submerged liquid cultures on a modified Kirk basal medium (MK), amended with 3.5 mM $CuSO_4$ and 3 % ethanol, as chemical laccase inducers and on Bran-Flakes medium (BF), containing natural LME inducers. Under these conditions maxima volumetric productions were reached in both media after 14-16 days (Figure 2). As laccase titers on MK media were about thrice higher than that on BF media (7.5 U ml^{-1} vs 2.3 U min^{-1}), it was selected for enzyme production in purification assays. Consistently with other reports on LME production by *Pycnoporus* species, LiP and MnP were not detected [5, 16, 30, 31, 32].

Figure 1. Fruit bodies (carpophores), mycelium colony and submerged culture of *Pycnoporus sanguineus* CS 2. Fungus identification was performed according to macroscopic and microscopic features. Strain isolation was done by tissue transference from the inner flesh of carpophores using mycological standard methodologies. Develop of orange-red pigmented mycelium on the edge of the solid plate colonies, and extracellular production of a reddish pigment under submerged conditions, were indicative of a successful isolation. Production of extracellular mucilage was also observed on submerged cultures. Isolation and identification details are given in text.

Laccase purification was started from about 1850 ml of mycelium-free filtrates from 14 day-old submerged cultures. After 10K ultraconcentration and sequential steps of anionic exchange chromatography on DEAE- Sepharose, gel filtration on Biogel P-100, and anionic exchange on Q-Sepahrose, laccase activity eluted as an apparently single protein peak with 100-140 mM phosphate (Figure 3). When aliquots of pooled laccase from this last chromatographic step were analyzed by denaturing SDS-PAGE, multiple protein bands were detected by Coomassie

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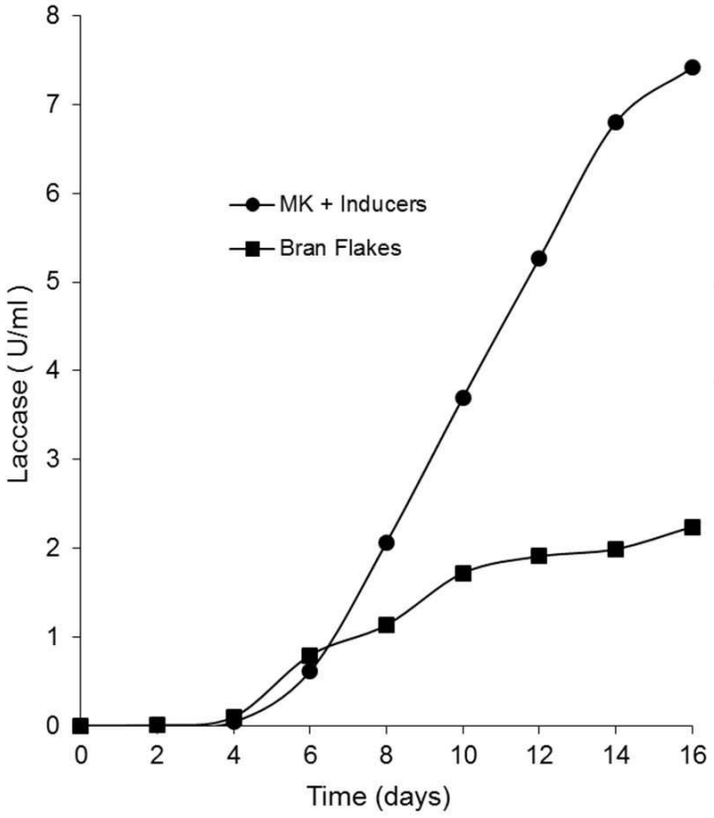

Figure 2. Time course of laccase production by *Pycnoporus sanguineus* CS2. Cultures were carried out on a modified Kirk basal medium, amended with 3.5 mM $CuSO_4$ (MK+ Inducers) and on Bran Flakes medium (BF) at 28 °C under 150 rpm agitation. Data represent the average of a representative assay in triplicate. Ethanol was added to MK medium at the third day of culture. Activity was determined with 2 mM 2, 6-DMP in 200 mM citrate-phosphate buffer pH 4.0

staining (not shown). A similar effect was reported in a work with a *Fusarium proliferum* laccase. As the multiband effect persisted after SDS substitution by other detergents, but disappeared in the absence of SDS, this phenomenon was associated to the presence of detergent on denaturing PAGE [33]. However, when we applied both a heat denatured sample and a non-boiled sample in parallel using the same SDS gel, a protein multiband and a single band were detected by Coomassie staining, respectively. Furthermore, the simple band pattern was also obtained in duplicates by activity staining using laccase substrates. This indicates that thermal treatment could be responsible of the observed multiband effect on denaturing conditions, instead of the SDS by itself. A summary of purification data is shown in Table 1. By this procedure a 16.7-fold purification and activity recovery of 25.5%, with specific activity of 69 U mg^{-1} protein was achieved. Concentrated purified enzyme showed the blue color characteristic of multi-cupper oxidases.

3.2. Biochemical properties

Electrophoresis analysis indicated that *Pycnoporus sanguineus* CS 2 produced only one laccase under the conditions used in this study. According to non-denaturing SDS-PAGE this laccase is a monomeric protein with a molecular weight of 64.4 kDa, and activity staining of native

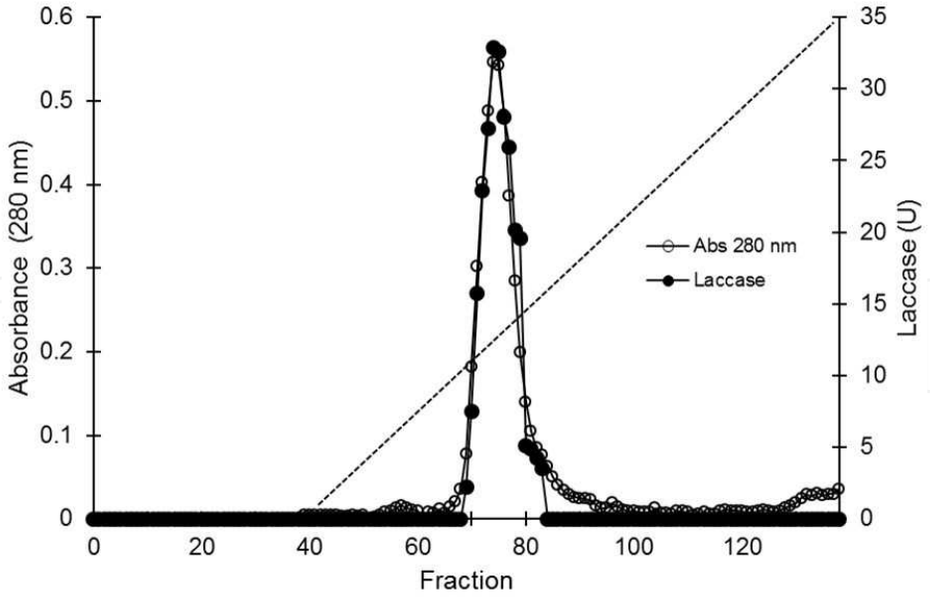

Figure 3. Elution profile for laccase from *Pycnoporus sanguineus* CS2 on anion-exchange column chromatography with Q-Sepharose (2.5 x 17 cm). The enzyme was eluted with a potassium phosphate (pH 6.0) linear gradient from 20-300 mM (dashed line) at a flow rate of 1.0 ml/min.

Purification step	Protein (mg)	Enzyme Activity (IU)	Specific Activity (U/mg)	Recovery (%)	Purification (fold)
Culture filtrate	1551	6477	4.18	100.0	1.0
Ultraconcentration 10 K	422	3724	8.8	57.4	2.1
DEAE-Sepharose FF	64	2308	35.8	35.6	8.5
Biogel P-100	32	1924	59.2	29.7	14.1
Q-Sepharose	22	1522	69.8	23.5	16.7

Table 1. Purification of *Pycnoporus sanguineus* CS 2 laccase

gels exhibited a single broad band when incubated with both, 2,6-DMP and the pair MBTH + DMAB, showing the same migration as the Coomassie blue stained band (Figure 4). Molecular mass of purified laccase was very similar to those reported for different *Pycnoporus sanguineus* strains [30, 31, 32], and it was consistent with the reported for most of basidiomycetes laccases [2, 3]. As expected for its visual appearance described above, the UV-Vis spectrum of purified enzyme was characteristic of the typical blue laccases, displaying the absorbance peak near to 600 nm related to the Cu-T1 centers, and the shoulder at 330 nm of Cu-T3 binuclear centers (Figure 5). Nonetheless, the oxidative coupling of MBTH and DMAB in the absence of mediators was indicative that *Pycnoporus sanguineus* CS2 laccase has the capability to catalyze reactions requiring a higher than usual redox potential for typical laccases [15].

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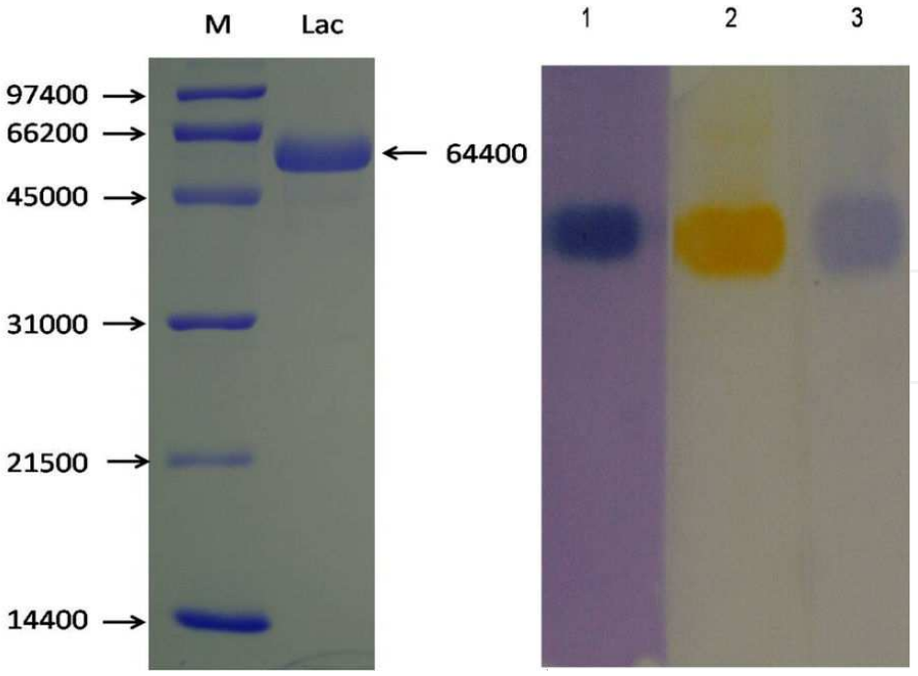

Figure 4. Electrophoresis analyses of purified *Pycnoporus sanguineus* CS 2 laccase by non-denaturing SDS- PAGE (left panel) and Native PAGE (right panel). Lanes M and Lac correspond to the Coomassie staining of molecular weight markers and purified laccase, respectively. The markers were phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4). On the right, lane 1 shows the Coomassie staining of purified laccase, and lanes 2 and 3, the activity staining with 2,6-DMP and the pair MBTH + DMAB, respectively.

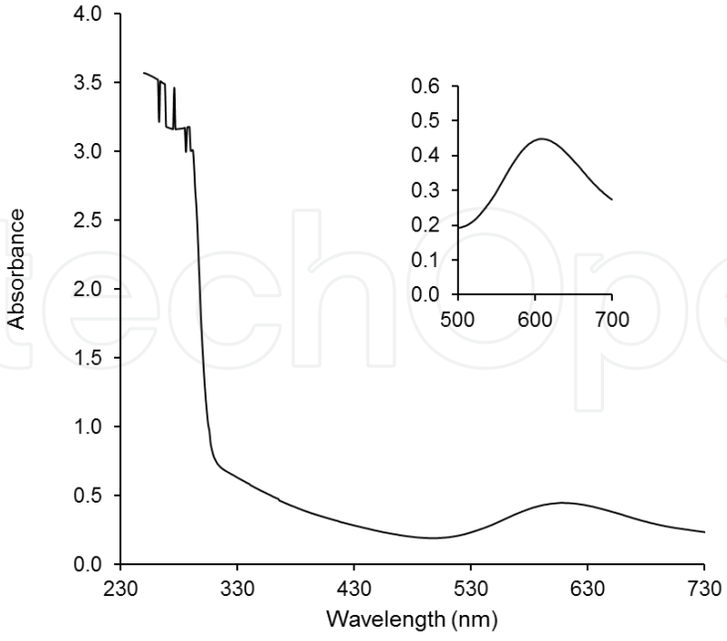

Figure 5. UV-Vis Spectrum of *Pycnoporus sanguineus* CS 2 Laccase. Assay was performed with a preparation of 25 µM laccase in bidistilled water. Insert shows the enlargement of the peak close to 610 nm.

In addition to blue laccases, other "atypical" forms of the enzyme named "yellow" laccases and "white" laccases have been reported. In the first case, it has been proposed that a variation in the redox state of Cu-T1 centers, by the presence of endogenous ligands, decreases the absorbance at 600 nm, without altering the spectral characteristics of the Cu-T2 and Cu-T3 centers, resulting in a yellow color [6]. In white laccases, like the one produced by *Pleurotus ostreatus*, it has been informed the presence of a single copper atom, which is accompanied by two of zinc and one of iron, instead of the regular four copper atoms [7]. In both, yellow and white laccases, the protein structure is similar to that found in blue laccases, but the changes in the redox state of the active site (whereas by the presence of the endogenous mediator or by the substitutions in the Cu centers), enables them to oxidize directly substrates of higher redox potential. According to all the above, the laccase produced by *P. sanguineus* CS 2 corresponds to a blue laccase, most likely containing the regular composition of Cu in its catalytic centers, but like atypical laccases is capable of acting on substrates of higher redox potential.

3.3. pH and temperature dependence

Enzyme was further characterized for its pH and temperature dependence. The effect of pH on laccase activity was studied using some of the most common laccase substrates, including the phenolic 2, 6-DMP, *o*-dianisidine and SGZ, as well as the non-phenolic ABTS. In general, laccase exhibited optima activity in the zone of pH between 3.0 and 4.5, depending on the particular substrate, then it declined in a gradual way towards the neutral zone of pH, and was completely lost at pH 6.5. Optimal pH values were 3.5, 3.5, 4.5 and 3.0 for 2, 6-DMP, *o*-dianisidine, SGZ and ABTS, respectively (Figure 6). These results were similar to those reported in literature for most of the fungal laccases [2]. It is known that biphasic pH-activity profiles with phenolic substrates (as the one showed by SGZ), are a consequence of two opposite effects: one generated by the difference in the redox potential between the reducer substrate and the Cu-T1 centers, when changing from acidic to neutral conditions. The other one is directly associated to the inhibitory action of OH^- ions over the activity of Cu-T2/T3 centers. For non-phenolic substrates as ABTS, the first effect should be minimal and the inhibition by OH^- reflects the monotonic decrease in the enzyme activity [12, 34].

The influence of temperature on *P. sanguineus* CS 2 laccase activity was investigated with 2, 6-DMP (2 mM), at pH 4.0, in the zone, from 20–80 °C. The profile temperature-activity showed a gradual increase from the lower limit at 20 °C to achieve an optimal value at 65 °C, and declined as temperature approached 80 °C. However, the enzymatic activity in these conditions remained relatively high compared to the value showed under optimal conditions (with a level close to 85%) (Figure 7). Indicating that *P. sanguineus* CS 2 laccase is a thermotolerant enzyme [35]. These data were evaluated according to the Arrhenius model in order to estimate the energy of activation (E_a) for the system. This parameter has been relatively little studied in thermotolerant laccases. The calculated E_a value (16.2 kJ/mol) for *P. sanguineus* CS 2 laccase is similar to the values reported for other thermotolerant laccases, as that for *Myceliophora thermophila* (19 kJ/mol) [36] and for the recombinant laccase from *Coprinus cinereus* (14 kJ/mol) [37], but smaller than those calculated in this report for other laccases, which apparently did

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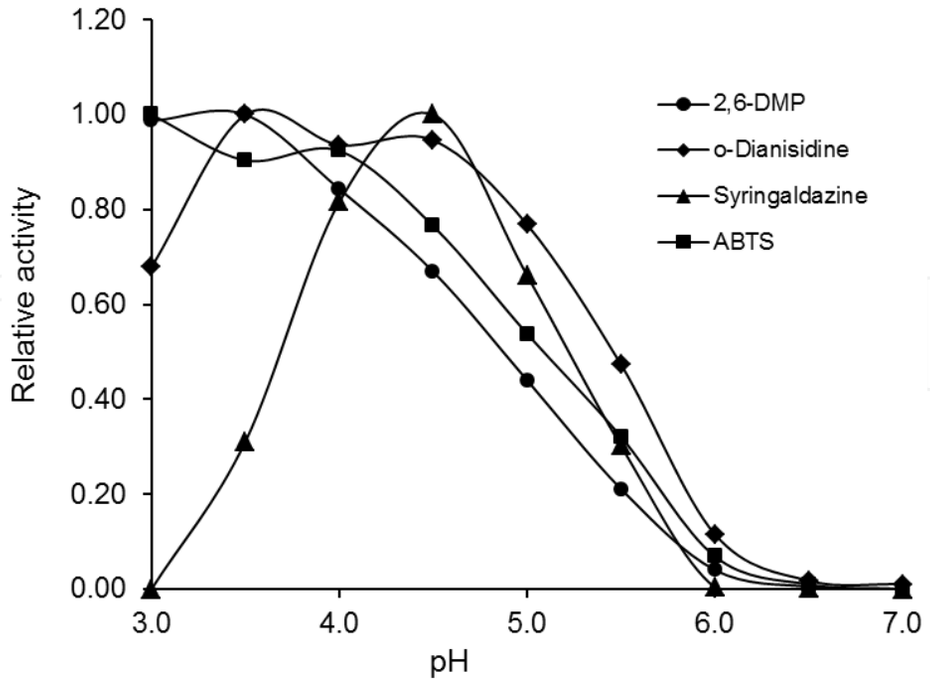


Figure 6. pH versus activity profiles of *Pycnoporus sanguineus* CS 2 laccase on various substrates. Assays were done by triplicate in 200 mM citrate-phosphate buffer at indicated pH, with 2, 6-DMP (2 mM), *o*-dianisidine (0.66 mM), SGZ (0.05 mM) and ABTS (2 mM).

not show a direct relationship between thermotolerance and the magnitude of E_a. On the other hand, the function showed a change in slope in the high temperature zone (50-70 °C) before the enzyme denaturing breaking zone. This effect could correspond to a decrease in the E_a of the system, caused by a thermotropic transition of the protein conformation, which should facilitate the limiting step of the reaction. Other possibility would be the coexistence of two enzyme populations, one of them showing an increased activity by temperature and the other being totally inactivated by thermal denaturing. These alternatives should be further explored.

Thermostability is a desirable property of industrial enzymes. Curves of temperature-stability of *P. sanguineus* CS 2 laccase showed that enzyme retained practically all of its activity after incubation for 8 h at 50 and 60 °C. Moreover, when incubations at 60 °C were extended to 24 h, the laccase retained 98% of its original activity (not shown). The enzyme also retained almost 50% of its activity after 4h at 70 °C. Inactivation curve showed a first-order decaying behavior (correlation > 0.96), with a calculated half-life ($t_{1/2}$) of 3.85 h [corresponding to a constant of thermal inactivation (k) of 0.187 h^{-1}]. To the best of our knowledge, this is one of the highest $t_{1/2}$ values found in laccases from mesophilic fungi. It is known that most of typical fungal laccases lose their activity in a few minutes at 60 °C [3, 15, 30, 38].

In comparison to laccases isolated from other *Pycnoporus* species, $t_{1/2}$ value at 70 °C here described is higher than those reported for laccase I (0.13 h) and laccase II (2.06 h) from *Pycnoporus* sp SYBC-L1 [13, 30], and for the laccase from the thermotolerant *P. sanguineus* CeIBMD001 (0.21 h) [16]. Native laccase also seems to be more resistant to thermal inactivation than *P. sanguineus* SCC 108 laccase ($t_{1/2}$ = 3.33 h at 65 °C) reported by [31] and the *P. sangui-*

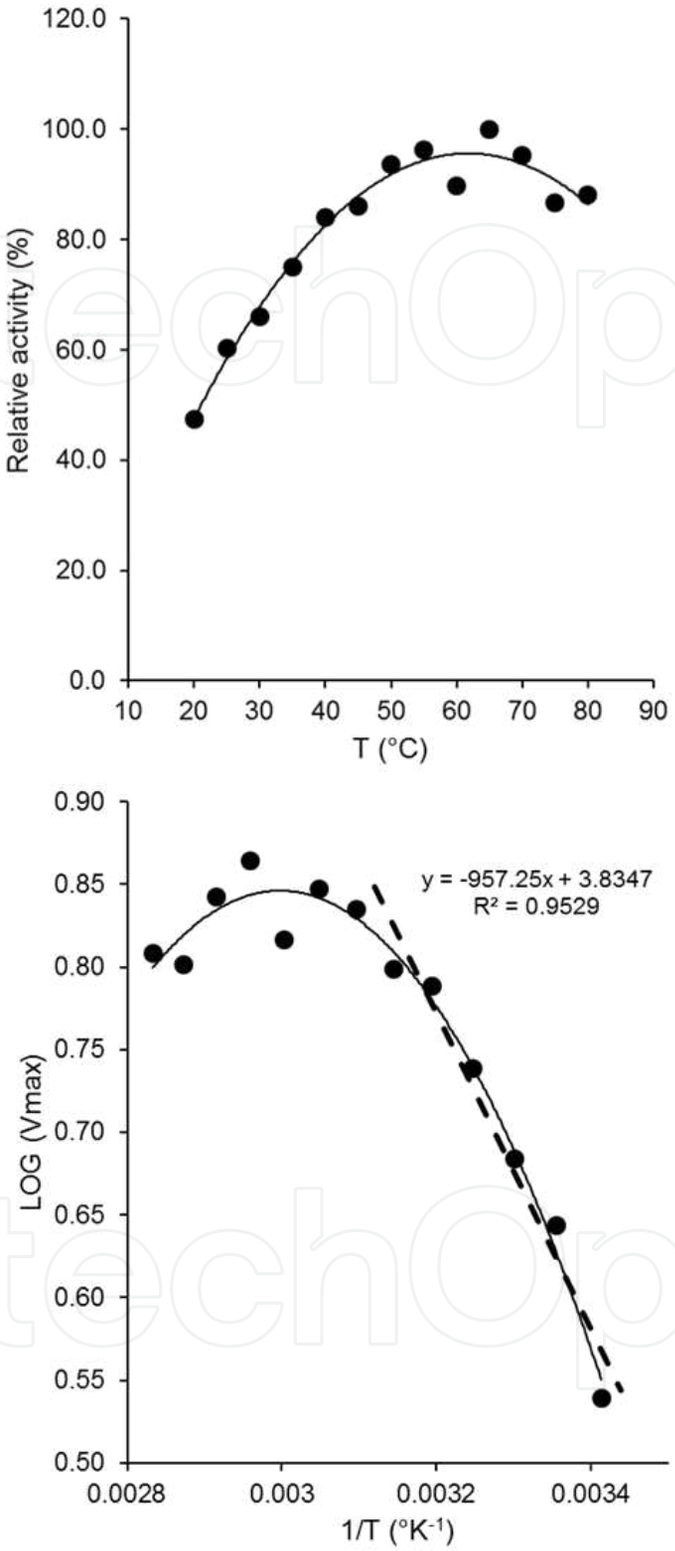


Figure 7. Effect of temperature on *Pycnoporus sanguineus* CS 2 laccase activity. Assays were performed by triplicate in 200 mM citrate-phosphate buffer at pH 4.0. Reaction rates were measured under saturating substrate concentrations (2 mM 2,6-DMP). The fitting line in lower panel shows the results of the Arrhenius analysis of data, corresponding to: LOG (Vmax) = [(-E_a /2.303 RT) + constant].

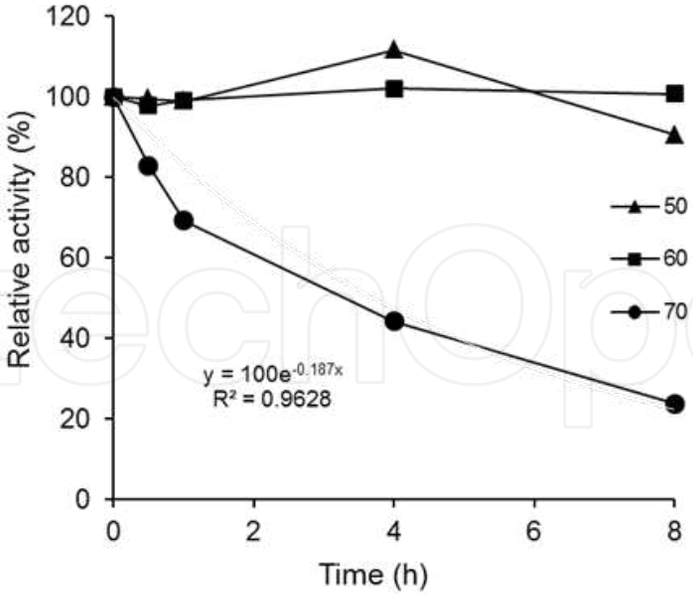


Figure 8. Effect of temperature on *Pycnoporus sanguineus* CS 2 laccase stability. Incubations were performed at various temperatures in distilled water. Aliquots were withdrawn at the indicated times and initial rates measured at 25 °C in 0.2 mM citrate/phosphate buffer at pH 4.0 with 2 mM 2, 6-DMP.

neus CCT- 4518 laccase studied in [39], which lost 60% of its initial activity after 2 h at 70 °C. Interestingly, three laccases from tropical or subtropical strains of *Pycnoporus* species (*P. sanguineus* BRFM 902, *P. sanguineus* BRFM 66, and *P. coccineus* BRFM 938) of different geographic regions (French Guinea, China and Australia, respectively) with remarkable thermal resistance have been recently reported by a research group in France [13]. A relationship between *P. sanguineus* CS 2 laccase with these and other *Pycnoporus* laccases already described was established by comparing the aminoacid sequences of an internal protein fragment (peptides 2+3+4, Table 2) from the native laccase with those sequences deposited at GenBank. Aminoacid sequence of *P. sanguineus* CS 2 laccase showed 99 % similarity to *P. sanguineus* BRFM 902 laccase, 93% to *P. coccineus* BRFM 938 [13], *P. cinnabarinus* PM laccases [5], and *P. sanguineus* BRFM 66 laccase [13], but only 84 % to *Trametes cinnabarina* [40] and 71% to *P sanguineus* CeIBMD001 laccase [16]. These results highlight the importance of *Pycnoporus* species biodiversity for the prospection for new thermostable laccases.

3.4. Kinetic properties

The kinetic properties of enzyme were studied with some typical substrates. The values of the Michaelis constant (K_m), catalytic constant (K_{cat}) and specificity constant (K_{cat}/K_m), were calculated by the Lineweaver-Burk method. Laccase showed the highest affinity and molecular activity, on ABTS (K_m = 23 mM, K_{cat} = 221 s^{-1}) compared to *o*-dianisidine (K_m = 44 mM, K_{cat} = 197 s^{-1}), and 2, 6-DMP (K_m =41 mM, K_{cat} = 88 s^{-1}). So, in terms of catalytic efficiency the best substrate resulted ABTS (K_{cat}/K_m = 9.4 x 10^6 s^{-1} M^{-1}) followed by *o*-dianisidine (K_{cat}/K_m = 4.5 x 10^6 s^{-1} M^{-1}) and 2, 6-DMP (K_{cat}/K_m = 2.2 x 10^6 s^{-1} M^{-1}). These results are summarized in Table 3, comparing the values of specificity constants (K_{cat}/K_m) for these substrates with those reported

for other *Pycnoporus* laccases, the native enzyme showed higher values for all assayed substrates except for the reported laccase II from *Pycnoporus sp* SYBC-L1 [30]. Like typical laccases, the enzyme of *P. sanguineus* CS 2, showed activity on a variety of substrates, such as the phenolic 2, 6-DMP, *o*-dianisidine and SGZ, as well as the non-phenolic ABTS.

Peptide	Amino acid sequences
1	EAVVVNGITPAPLIAGKK
2*	GPFVVYDPNDPQASLYDIDNDDTVITLADWYHLAAKVGQR
3*	FPLGADATLINGLGR
4*	TPGTTSADLAVIKVTQGK
5	YSFVLDASQPVDNYWIRANPPFGNVGFAGGINSAILR
6	SAGSSEYNYDNPVFR

* Contiguous peptides of the internal laccase fragment used in alignments

Table 2. Amino acid sequences corresponding to internal peptides of *Pycnoporus sanguineus* CS2 laccase

Substrate	K_m (µM)	V_{max} (µmol/min/ml)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/M^{-1})
2,6-DMP	41	500	88	2.16×10^6
o-dianisidine	44	1111	197	4.49×10^6
ABTS	23	1250	221	9.38×10^6

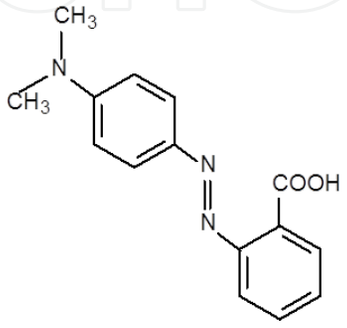
Table 3. Kinetics constants of *Pycnoporus sanguineus* CS 2 laccase

Among these substrates this laccase showed preference for ABTS and this characteristic was consistent with most of fungal laccases [32, 41, 42]. Unexpectedly the substrate saturation graphics with SYR showed a sigmoidal-like behavior instead of the common hyperbolic one (not shown). This result could be explained considering a kinetic mechanism of positive cooperativity as that described for monomeric mnemonical enzymes [43], where a conformational change of interacting enzyme at the end of the first catalytic cycle, reacts more readily with a second substrate molecule than other free-enzyme. Other factor contributing to this result could be the presence of ethanol in routinely SYR assay affecting the substrate solubility and/or enzyme activity. Whether mechanistic on phenomenological, this observation must be taken into account in future works, considering the relevance of this substrate in laccase characterization.

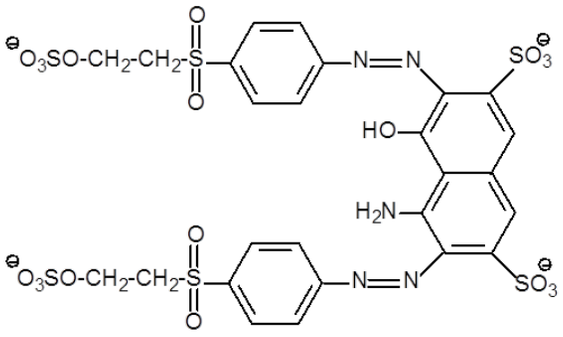
3.5. Dye decolorization

As revealed by the activity staining of native gels shown above, *P. sanguineus* CS 2 laccase was also able to promote the oxidative coupling between MBTH and DMAB. This reac-

tion has been considered as indicative of the ability of some laccases to catalyze reactions requiring a higher redox potential, as in the case of the enzymatic decolorization of many synthetic dyes. The non-phenolic azo MR [44, 45] and diazo RB 5 dyes [46] have been used as models for studying the ability of laccases to degrade recalcitrant compounds (Figure 9). Although general consensus is that laccases require meditators for acting over these dyes, *P. sanguineus* CS 2 laccase showed the capability to decolorize directly both compounds, but with different ability. Decolorization of MR and RB 5 reached a level of 70 %, and 15% respectively, after 4 h at 25 °C.



Methyl Red (Acid Red 2, CI: 13020)



Reactive Black 5 (RB 5, CI: 20505)

Figure 9. Chemical structure of the recalcitrant methyl red and reactive black 5 dyes used in this study.

While *Ganoderma lucidum* [38], *Trametes trogii* [47] and *Lentinula edodes* [45] laccases were only able to decolorize RB 5 in the presence of mediators, a recent report state that three *Pycnoporus* laccases [13], were able to perform this decolorization in the absence of mediators, under similar conditions used in this work, with decolorization reaching from 29 to 45% after 52 h, at room temperature. The recalcitrance of RB 5 to laccase decolorization has been explained by its high redox potential or steric hindrances limiting accessibility of enzyme to –OH and $–NH_2$ groups in dye. As in this study native laccase attained around 70% decolorization after 20 h at room temperature, decolorization assays were performed at 60 °C taking advantage of its thermostability trying to overcome limiting factors. As expected, decolorization process was faster under the influence of tempera-

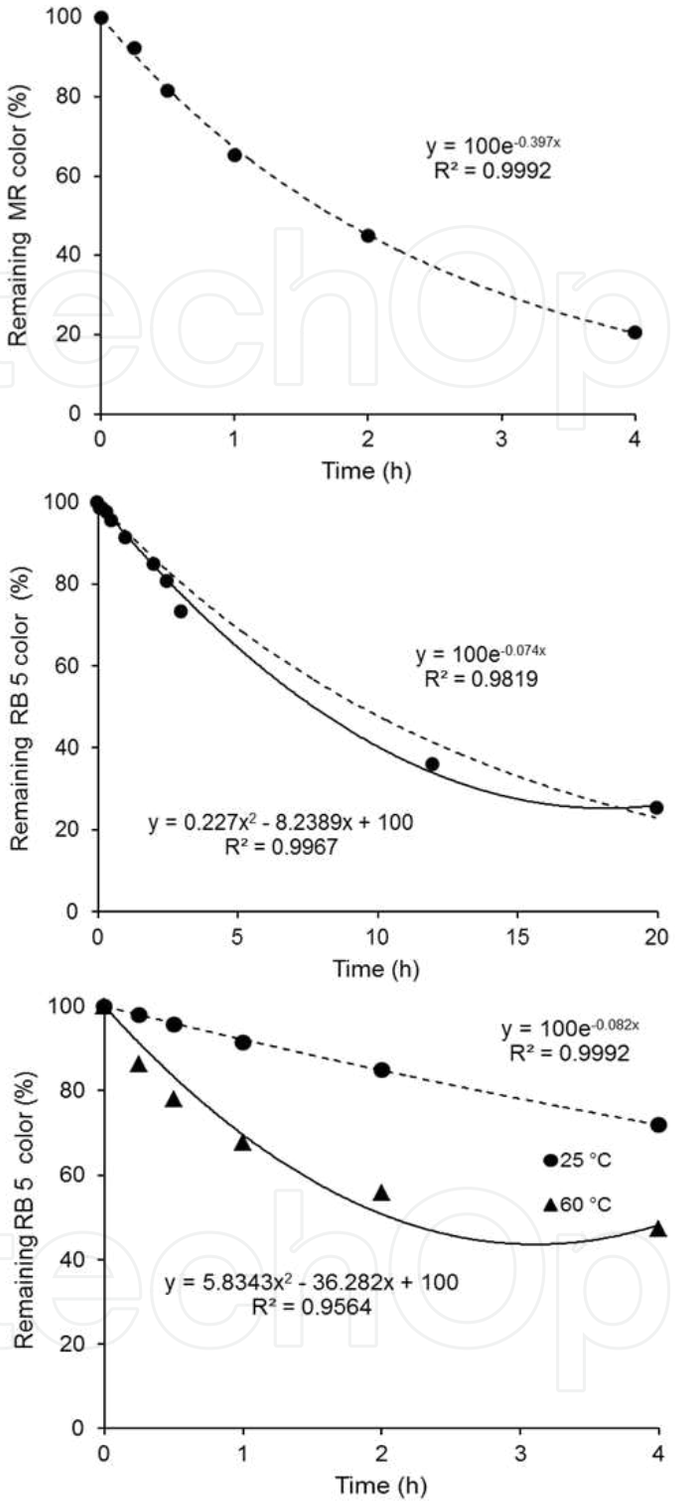


Figure 10. Decolorization of methyl red and reactive black 5 by *Pycnoporus sanguineus* CS2 laccase. Assays were performed by incubating 25 μM Methyl Red (upper panel) and 25 μM Reactive Black 5 (middle and lower panels) with laccase (5U/ml) in 0.2 mM citrate/phosphate buffer at pH 4.0 Aliquots were withdrawn from the assay mixture at the indicated times and remaining color was determined as described in text. Lines showed the best data fittings corresponding to the exponential first-order (dashed) or polynomial second-order decay functions (continuous).

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ture, reaching around 50% after 4 h, although it also seems to be limited faster (Figure 10). While MR decolorization fitted an exponential first order decay model, RB 5 decolorization changes rapidly from this behavior to fit a polynomial second order model. This effect could be related to several factors as an increased enzyme inactivation by endogenous generated reaction intermediates and/or dead-end transformation products. This relationship must be investigated in future work. Nonetheless these results illustrate the potential of the thermostable *Pycnoporus sanguineus* CS2 laccase for practical applications.

4. Conclusion and future prospects

Its thermostability and ability for acting on high redox substrates and recalcitrant dyes, makes *Pycnoporus sanguineus* CS 2 laccase a good prospect for its application in industrial and environmental processes. This laccase could also be interesting as a model in studies associating structure-function of thermotolerant proteins from mesophilic microorganisms.

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References

[1] Mayer AM, Staples RC. Laccase: New Functions for an Old Enzyme. Phytochemistry 2002;60 551-565.

[2] Baldrian P. Fungal Laccases-Occurrence and Properties. FEMS Microbiological Reviews 2005;20 1-28.

[3] Morozova OV, Shumakovich GP, Gorbacheva MA, Shleev SV, Yaropolov AI. ''Blue'' Laccases. Biochemistry (Moscow) 2007;72 1136–1412.

[4] Thurston CF. The Structure of Fungal Laccases. Microbiology 1994 ;140 19-26.

[5] Eggert C, Temp U, Eriksson K-EL. The Ligninolytic System of the White- rot Fungus *Pycnoporus cinnabarinus*: Purification and Characterization of the Laccase. Applied Environmental Microbiology 1996;62 1151–1158.

[6] Leontievsky AA, Vares T, Lankinen P, Shergill JK, Pozdnyakona NN, Myasoedova NM. Blue and Yellow Laccases of Ligninolytic Fungi. FEMS Microbiological Letters 1997;156 9-14.

[7] Palmieri G, Giardina, P, Bianco C, Sacloni A, Capasso A, Sannia G.A A Novel White Laccase from *Pleurotus ostreatus*. Journal of Biological Chemistry 1997;50 31301-31307.

[8] Pointing SB. Feasibility of Bioremediation by White–rot Fungi. Applied Microbiology and Biotechnology 2001;57 20-33.

[9] Reddy CA. The Potential of White-rot Fungi in the Treatment of Pollutants. Current Opinion in Biotechnology 1995;6 320-328.

[10] Rodríguez-Couto S, Toca-Herrera JL. Laccases in the Textile Industry. Biotechnology and Molecular Biolology Reviews 2006a;1 115-120.

[11] Rodríguez-Couto S, Toca-Herrera JL. Industrial and Biotechnological Applications of Laccases: A review. Biotechnology Advances 2006b;24 500-513.

[12] Xu F, Berka RM, Wahleithner JA, Nelson BA, Shuster JR, Brown SH, Palmer AE, Solomon EI. Site-directed Mutations in Fungal Laccase: Effect on Redox Potential, Activity and pH Profile. Biochemistry Journal 1998;334(1) 63-70.

[13] Uzan E, Nousiainen P, Balland V, Sipila J, Piumi F, Navarro D, Asther M, Record E, Lomascolo A. High Redox Potential Laccases from the Ligninolytic Fungi *Pycnoporus coccineus* and *Pycnoporus sanguineus* Suitable for White Biotechnology: from Gene Cloning to Enzyme Characterization and Applications. Journal of Applied Microbiology 2010;108 2199-2213.

[14] Rodgers CJ, Blanford CF, Giddens SR, Skamnioti P, Armstrong FA Gurr SJ. Designer Laccases: A Vogue for High-potential Fungal Enzymes?. Trends in Biotechnology 2009;28(2) 63-72.

[15] Jordaan J, Leukes WD. Isolation of a Thermostable Laccase with DMAB and MBTH Oxidative Coupling Activity from a Mesophilic White-rot Fungus. Enzyme and Microbial Technology 2003;33 212-219.

[16] Dantán-González E, Vite-Vallejo O, Martínez-Anaya C, Méndez-Sánchez M, González MC, Palomares LA, Folch-Mallol J. Production of Two Novel Laccase Isoforms by a Thermotolerant Strain of *Pycnoporus sanguineus* Isolated from an Oil-polluted Tropical Habitat. International Microbiology 2008;11 163–169.

[17] Pozdnyakova NN, Turkovskaya OV, Yudina EN Rodakiewicz-Nowak Y. Yellow Laccase from the Fungus *Pleurotus ostreatus* D1: Purification and Characterization. Applied Biochemistry and Microbiology 2006;42(1) 56-61.

[18] Hernández-Luna CE, Gutiérrez-Soto G, Salcedo-Martínez SM. Screening for Decolorizing Basidiomycetes in Mexico. World Journal of Microbiology and Biotecnology 2008;24 465-473.

[19] Ryvarden L. Genera of Polypores Nomenclature and Taxonomy. Synopsis fungorum Vol. 5. Fungiflora A/S Oslo; 1991.

[20] Guzmán G. Identificación de los Hongos: Comestibles, Venenosos, Alucinantes y Destructores de la Madera: Editorial Limusa S.A. México; 1980.

[21] Abadulla E, Tzanov T, Costa S, Robra K, Gübitz G. Decolorization and Detoxification of Textile Dyes with a Laccase from *Trametes hirsuta*. Applied and Environmental Microbiology 2000;66 3357-3362.

[22] Tien M, Kirk TK. Lignin Peroxidase of *Phanerochaete chrysosporium*. Methods in Enzymology 1988;161 238-248.

[23] Kuan C, Johnson J, Tien M. Kinetic Analysis of Manganese Peroxidase. The Reaction with Manganese Complexes. Journal of Biological Chemistry 1993;268 20064–20070

[24] Pickard MA, Vandertol H, Roman R, Vazquez-Duhalt R. High Production of Ligninolytic Enzymes from White-rot Fungi in Cereal Bran Flakes Liquid Medium. Canadian Journal of Microbioliology 1999;45 627-631.

[25] Dhouib A, Hamza M, Zouari H, Mechichi T, Hmidi R, Labat M, Martinez MJ, Sayadi S. Screening for Ligninolytic Enzyme Production by Diverse Fungi from Tunisia. World Journal of Microbiology and Biotechnology 2005 ;21 1415-1423.

[26] Zouari-Mechichi H, Mechichi T, Dhouib A, Sayadi S, Martínez AT, Martínez MJ. Laccase Purification and Characterization from *Trametes trogii* isolated in Tunisia: Decolorization of Textile Dyes by the Purified Enzyme. Enzyme and Microbial Technology 2006;39 141-148.

[27] Laemmli U. Cleavage of Structural Proteins During the Assembly of the Head of the Bacteriophage T4. Nature 1970;227 680-685.

[28] Garfin DE. Methods in Enzymology. Academic Press Inc. 1990.

[29] Guzmán G. Los Hongos del Edén Quintana Roo. (*Introducción a la Micología Tropical de México*). INECOL y CONABIO, Xalapa ; 2003.

[30] Wang ZX, Caia YJ, Liao XR, Taoc GJ, Lia YY, Zhanga F, Zhang DB. Purification and Characterization of Two Thermostable Laccases with High Cold Adapted Characteristics from *Pycnoporus* sp. SYBC-L1. Process Biochemistry 2010;45 1720-1729.

[31] Litthauer D, Jansen van Vuuren M, van Tonder A, Wolfaardt FW. Purification and Kinetics of a Thermostable Laccase from *Pycnoporus sanguineus* (SCC 108). Enzyme Microbial Technology 2007;40 563–568.

[32] Lu L, Zhao M, Zhang BB, Yu SY, Bian X-J, Wang W, Wang Y. Purification and Characterization of Laccase from *Pycnoporus sanguineus* and Decolorization of an Anthraquinone Dye by the Enzyme. Applied Microbiology and Biotechnology 2007;74 1232-1239.

[33] Hernández-Fernaud JR, Marina A, González K, Vázquez J, Falcón MA. Production, Partial Characterization and Spectroscopic Study of the Extracellular Laccase Activity from *Fusarium proliferatum.* Applied Microbiology and Biotechnology 2006 ;70 212-221.

[34] Madzak C, Mimmi MC, Caminade E, Brault A, Baumberger S, Briozzo P, Mougin C, Jolivalt C. Shifting the Optimal pH of Activity for a Laccase from the Fungus *Trametes versicolor* by Structure-based Mutagenesis. Protein Engineering 2006;334 63-70.

[35] Hildén K, Hakala TK, Lundell T. Thermotolerant and Thermostable Laccases. Biotechnological Letters 2009;31 1117–1128.

[36] Holm KA, Nielsen DM, Eriksen J. Automated Colorimetric Determination of Recombinant Fungal Laccase Activity in Fermentation Samples Using Syringaldazine as Chromogenic Substrate. Journal of Automated Chemistry 1998;20 199 –203.

[37] Schneider K, Caspersen MB, Mondorf K, Halkier T, Skov LK, Ostergaard PR, Brown KM, Brown SH, Xu F. Characterization of a *Coprinus cinereus* Laccase. Enzyme and Microbial Technology 1999;25 502–508.

[38] Murugesan K, Nam IH, Kim YM, Chang YS. Decolorization of Reactive Dyes by a Thermostable Laccase Produced by *Ganoderma lucidum* in Solid-state Culture. Enzyme and Microbial Technology 2007;40 1662-1672.

[39] García TA, Santiago MF, Ulhoa CJ. Studies on the *Pycnoporus sanguineus* CCT-4518 Laccase Purified by Hydrophobic Interaction Chromatography. Applied Microbiology and Biotechnology 2007;75 311–318.

[40] Antorini M, Herpoel-Gimbert I, Choinowski T, Sigoillot JC, Asther M, Winterhalter K, Piontek K. Purification, Crystallization and X-ray Diffraction Study of Fully Functional Laccases from Two Ligninolytic Fungi. Biochemistry and Biophysics Acta 2002;1594 (1) 109-114

[41] Ko EM, Leem YE, Choi HT. Purification and Characterization of Laccase Isoenzymes from the White-rot Basidiomycete *Ganoderma lucidum*. Applied Microbiology and Biotechnology 2001;57 98-102.

[42] Zang H, Zang Y, Huang F, Gao P, Chen J. Purification and Characterization of a Thermostable Laccase with Unique Oxidative Characteristics from *Trametes hirsuta*. Biotechnology Letters 2009;31 837-843.

[43] Ricard J, Noat G. Kinetic Co-operativity of Monomeric Mnemonical Enzymes 'The Significance of the Kinetic Hill- coefficient. European Journal of Biochemistry 1985;152 557-564.

[44] Haibo Z, Yinglong Z, Feng H, Peiji G, Jiachuan C. Purification and Characterization of a Thermostable Laccase with Unique Oxidative Characteristics from *Trametes hirsuta*. Biotechnological Letters 2009;31 837-843.

[45] Nagai M, Sato T, Watanabe H, Saito K, Kawata M, Enei H. Purification and Characterization of an Extracellular Laccase from the Edible Mushroom *Lentinula edodes*, and Decolorization of Chemically Different Dyes. Applied Microbiology and Biotechnology 2002;60 327-335.

[46] Camarero S, Ibarra D, Martínez MJ, Martínez AT. Lignin-Derived compounds as Efficient Laccase Mediators for Decolorization of Different Types of Recalcitrant Dyes. Applied and Environmental Microbiology 2005;71(4) 1775-1784.

[47] Zeng X, Cai Y, Liao X, Zeng X, Li W, Zhang D. Decolorization of Synthetic Dyes by Crude Laccase from a Newly Isolated *Trametes trogii* Strain Cultivated on Solid Agro-industrial Residue. Journal of Hazardous Materials 2011;189 517-525.

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