Production of two novel laccase isoforms by a thermotolerant strain of *Pycnoporus sanguineus* isolated from an oil-polluted tropical habitat

Edgar Dantán-González,† Odón Vite-Vallejo,† Claudia Martínez-Anaya,† Mónica Méndez-Sánchez,‡ María C. González,³ Laura A. Palomares,⁴ Jorge Folch-Mallol†*

†Biotechnology Research Center, Autonomous University of the State of Morelos, Cuernavaca. ‡Faculty of Biological Sciences, Autonomous University of the State of Morelos, Cuernavaca. †Institute of Biology, National Autonomous University of Mexico, Mexico, D.F. ⁴Biotechnology Institute, National Autonomous University of Mexico, Cuernavaca, Mexico

Received 10 March 2008 · Accepted 15 June 2008

**Summary.** A thermotolerant and halotolerant strain of *Pycnoporus sanguineus* was isolated from an oil-polluted site in a tropical area located in Veracruz, Mexico. This strain was able to grow at 47°C and in culture medium containing 500 mM NaCl. The strain was also tolerant to the presence of 30,000 ppm of crude Maya oil. A 68-kDa protein purified from submerged cultures exhibited laccase activity towards 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), guaiacol, syringaldazine, and *o-*dianisidine, for which it presented the highest affinity (*K_m* = 43 μM). Two-dimensional gel electrophoresis analysis showed that, unusual for laccases, the enzyme has two active isoforms, with isoelectric points of 7.00 and 7.08. The purified enzyme showed high thermostability, retaining 40% of its original activity after 3 h at 60°C. This property seems to correlate with a long “shelf-life,” given that at 40°C enzyme activity was only gradually lost over a 5-day period incubation. Both the fungus and its laccase are likely to have high potential for biotechnological applications. [Int Microbiol 2008; 11(3):163-169]

**Key words:** *Pycnoporus sanguineus* · *Bjerkandera adusta* · Basidiomycota · laccases · thermotolerant fungi · bioremediation

**Introduction**

Filamentous fungi of the phylum Basidiomycota have been widely studied for their ability to degrade wood [13]. Lignin, a major component of wood, is one of the most abundant polymers in nature. It consists of a heterogeneous and highly cross-linked polymer with structural similarity to several recalcitrant pollutants, such as polycyclic aromatic hydrocarbons (PAH), and several pesticides and industrial dyes [3]. Within the basidiomycetes, white-rot fungi have received special attention because they are capable of mineralizing lignin by secreting oxidative enzymes, such as peroxidases and laccases, which have a broad range of substrates [10]. Some ascomycete fungi are also capable of degrading lignin, xenobiotic compounds, and PAH [23,24]. *Botryosphaeria rhodina* produces only one kind of polyphenol oxidase, a laccase, that degrades the herbicide imazaquin [20] as well as many lignin-like compounds [5]. Laccases produced by *B. rhodina* MAMB-05 were described in detail in a recent work [6].

Laccases (EC 1.10.3.2) are multi-copper oxidases found in different groups of organisms (bacteria, fungi, plants, and insects) [17], although fungal laccases are the best studied. The enzymes are glycoproteins with molecular masses of 50–103 kDa and require oxygen to oxidize phenols, polyphe-
nols, aromatic amines, and other non-phenolic compounds. Substrate oxidation can, in turn, generate radicals that are capable of non-enzymatic oxidation of different substrate molecules. Radicals and other small molecules are called mediators and they contribute significantly to the degradation of non-phenolic compounds present in lignin [4,7,14,15]. Laccases have a broad range industrial applications, including decolorizing and detoxifying effluents, drug analysis, textile-dye bleaching, polymer syntheses, and bioremediation [17].

_Pycnoporus cinnabarinus_ is an interesting laccase-producing species because of its simple ligninolytic system capable of mineralizing lignin. Neither lignin peroxidase nor manganese peroxidase activities have been detected in this species, but its laccase has been well characterized [8]. Two laccase genes have been cloned from _P. cinnabarinus_, _lcc3-1_, or the allelic form _lac1_ [9], and _lcc3-2_ [25]. In _Pycnoporus sanguineus_, three laccase isoforms with different molecular masses have been reported [11,16] and at least two of them are thermostable [12,16]. These three laccase isoforms from the same organism were identified under different growing conditions. Garcia et al. [11,12] used 2,5-xylidine as an inducer in malt extract medium, while Littthauer et al. [16] grew _P. sanguineus_ in the presence of molasses. All the isoforms have different molecular masses and kinetic parameters. This highlights the importance of the growing conditions of the fungus and/or reflects the genetic differences of each strain. In this work, we report the isolation and characterization of a thermostolerant and halotolerant strain of _P. sanguineus_, as well as the characterization of its laccase activity. Our findings are compared with those described in previous reports. The results suggest that different strains of the same species produce laccases with different properties and characteristics.

### Materials and methods

**Isolation of a thermostolerant strain of _Pycnoporus sanguineus_.** Fruiting bodies of an orange fungus growing on dead tree bark covered by an oil spill were collected. Pieces of the basidiocarp were sterilized and propagated in V8 medium (18% Campbell V8 vegetable juice, 2% agar, and 0.2% CaCO₃) containing 240 U penicillin/ml. After successive passages, axenic cultures were identified morphologically and microscopically 

Genomic DNA isolation and other molecular techniques. Genomic DNA was isolated, using the UltraClean Soil DNA kit (Mo Bio, Carlsbad, CA), from 7-day-old mycelium grown on glucose-maltose yeast extract (GMY) medium (1% glucose, 0.35% malt extract, 0.25% yeast extract, 0.2% KH₂PO₄, and 0.05% MgSO₄·7H₂O, pH 4.5, adjusted with phosphoric acid). Oligonucleotides for the amplification of a fragment of the 18S rDNA were previously reported [2]. DNA was sequenced in an automated sequencer (model ABI Prism 377-18; Applied Biosystems) with the ABI Prism BigDye Terminator cycle sequencing Ready Reaction kit.

**Thermostolerance, halotolerance, and oil-tolerance assays.** These tests were carried out on Petri dishes containing 25 ml of solid GMY. _Bjerkandera adusta_ strain UAMH 8258 served as the reference species (kindly donated by Dr. Rafael Vázquez-Duhalt). Mycelia were obtained from starter cultures grown on GMY for 5 days at 28°C. For thermostolerance assays, 0.16-cm² squares from the starter culture were placed on a fresh plate and incubated for an additional 24 h at 28°C before the cultures were transferred to the experimental conditions (28, 37, 42, 45, and 50°C). Growth was quantified daily by measuring the diameter of the colonies. Similarly, halotolerance was assayed by the addition of 500 mM NaCl to the media. These cultures were incubated at 28°C (_B. adusta_) or 37°C (_P. sanguineus_). Finally, the ability to grow in the presence of oil was tested on GMY medium containing 0, 10,000, or 30,000 parts per million (ppm) of crude Mayan oil (supplemented with 0.25% of soybean lecithin as emulsifier), incubating cultures of each species at their temperature optima. Maya oil was previously described [Boletín del Instituto de Investigaciones Electrécicas May/June, 1998; http://www.iee.org.mx/publica/bolmj98/secm98.htm] as a dense Mexican oil of 22 API (American Petroleum Institute) gravity degrees and an asphaltene content of 11–14%, with significant amounts of nickel (53 ppm) and vanadium (298 ppm).

**Laccase production and activity assays.** _Pycnoporus sanguineus_ was inoculated in 125-ml flasks containing 75 ml of bran flaxes (BF) medium (3% ground Kellogg’s Bran Flakes, in 60 mM phosphate buffer pH 6 [26]) and incubated at 200 rpm and 28°C for 14 days. Bran Flakes contain 33% sugars, 34% starch, and 13% fiber, according to the manufacturer’s information and it has been successfully used for the inductions of ligninolytic enzymes [26]. Laccase activity was monitored daily by oxidation of 1 mM of 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in acetate buffer (pH 4.5), measuring formation of the cation radical (ε₄₅₀ = 2.9 × 10⁴ M⁻¹ cm⁻¹). On the day of maximum activity, supernatants were prepared by filtering the mycelium and then centrifuging the filtrate for 20 min at 5250 × g. Activity was also determined with guaiacol (ε₄₈₇ = 2.6 × 10⁴ M⁻¹ cm⁻¹), syringaldazine (ε₄₅₀ = 6.5 × 10⁴ M⁻¹ cm⁻¹), and o-dianisidine (ε₄₅₀ = 1.1 × 10⁴ M⁻¹ cm⁻¹). One unit of laccase activity is defined as the amount of enzyme required to oxidize 1 μmol of substrate per unit of time (min).

**Purification of laccase.** Four volumes of cold acetone were added to culture supernatants. The mixture was incubated 1.5 h at −20°C and then centrifuged 30 min at 10,000 × g; the pellet was dissolved in 1/10 volume of water. The enzyme was purified by gel chromatography using a Sepharose High Traps Q column (Amersham) equilibrated with acetate buffer 25 mM (pH 5.5), and then elution with a gradient of 1 M NaCl (0–30%) at a flow rate of 1 ml/min. Fractions containing the highest laccase activity were pooled, desalted, and concentrated by ultrafiltration (Centricron, Millipore; 30-kDa exclusion).

**Enzymatic temperature stability.** The amount of purified laccase activity remaining after incubation at temperatures ranging from 40 to 70°C was measured during the times stated below. Determinations were carried out in triplicate.

**Gel electrophoresis.** Laccase purity was determined by Coomassie staining after SDS-PAGE. Molecular mass was established by comparison with molecular mass markers (BenchMark Protein ladder, Invitrogen). To detect laccase activity in gels, samples (without 2-mercaptoethanol and prior to boiling) were analyzed by SDS-PAGE and then incubated with 1 mM ABTS or 2.5 mM 2,6-DMP (dimethoxyphenol) in 100 mM acetate buffer (pH 4.5). Isoelectric points (pI) of the laccase isoforms were determined by two-dimensional (2D) gels (IEF-PAGE). Gels were prepared using ampholytes of pH 3–10, and 4–6. pI values were determined by measuring the pH in parallel IEF gels. Duplicate 2D gels were stained either for protein with Coomassie BlueW or for laccase activity.
Calculation of kinetic parameters. Fungal growth rate was calculated from the slope of the growth curve. The enzymatic inactivation constant (kd) of P. sanguineus laccase at different temperatures was determined by adjusting the data to a first-order decay model \( \frac{dA_c}{dt} = kdA_c \), where \( A_c \) is residual activity (%) and \( t \), time (h). The adjusted data had a correlation >0.96 at all temperatures. The \( t_{1/2} \) at different temperatures was calculated as \( t_{1/2} = \ln 2/kd \).

Results

Isolation of a thermotolerant, halotolerant strain of Pycnoporus sanguineus from an oil spill. A fungus found growing on top of an oil spill near a refinery was collected in the southern portion of the state of Veracruz, Mexico (18° 06′ N, 94° 24′ W). Through the use of morphological and molecular techniques, this fungus was identified as a strain of Pycnoporus sanguineus. Alignment of a 750-bp fragment of 18S rDNA, amplified by PCR using primers specific for fungi [2] (GenBank accession no. EU000253) showed 99% identity with accession number AY705970, which corresponds to a well-characterized Pycnoporus strain. We named the strain CeIBMD001.

It was reasoned that, because of its tropical origin, P. sanguineus should tolerate extreme physical conditions. Thermotolerance and halotolerance of this strain were established, and B. adusta, a species isolated from temperate forests, was used as reference strain. Figure 1A shows that P. sanguineus was able to grow at 47°C, in contrast to B. adusta. Furthermore, B. adusta died at 37°C, since after 9 days at this temperature it was unable to resume growth when Petri dishes containing the cultures were transferred back to 28°C. Growth of P. sanguineus was very slow at 50°C, however, after 9 days under this condition, growth of the fungus recovered when it was transferred back to 28°C (data not shown). The optimal growth temperature for P. sanguineus CeIBMD001 was 37°C.

Various concentrations of salt were also tested to assess the halotolerance of these fungi. Under control conditions in GMY, P. sanguineus grew faster than B. adusta (2.43 vs. 1.87 cm/day, respectively) which is an advantage for the production of biotechnologically relevant enzymes. Figure 1B is a comparison of the growth of P. sanguineus to B. adusta in 500 mM NaCl, a salt concentration similar to that found in seawater [http://www.waterencyclopedia.com/Mi-Oc/Ocean-Chemical-Processes.html]. Under this condition, B. adusta was unable to grow whereas P. sanguineus grew, although its growth rate decreased by 85% compared to growth in medium without salt (0.383 vs. 2.43 cm/day). Although the growth of P. sanguineus was modest at 500 mM NaCl, the halotolerance of this strain was nonetheless considerable, since B. adusta died at the same salt concentration.

The ability of these species to grow in the presence of oil was also investigated. The high content of asphaltenes and heavy metals in Mayan oil makes it particularly difficult to
degrade by microorganisms. Each strain was grown at its optimal temperature in a range of oil concentrations. Oil concentrations of 10,000 ppm reduced the growth of the two strains to the same extent (data not shown). However, in the presence of 30,000 ppm of crude Mayan oil *P. sanguineus* formed a thicker mycelium than *B. adusta*, indicative of higher biomass production as a result of a better growth under this condition (Fig. 2).

Altogether, these experiments indicated that *P. sanguineus* CelBMD001 is more capable of resisting harsher conditions than species from temperate climates.

**Laccase purification and characterization.** *Pycnoporus sanguineus* CelBMD001 was grown on BF medium and extracellular laccase activity was measured for 14 days.
Maximum enzyme activity was achieved at day 7 (6 U/ml), although considerable activity levels (~4 U/ml) were detected up to day 14 (data not shown). Laccase was purified from 7-day cultures. Crude extracts were obtained by acetone precipitation and laccase was purified by anion-exchange chromatography, with a recovery of 9.9%. Unbound proteins were separated with the first wash with buffer, after which a salt gradient resolved several peaks, the first of which, at 220 mM NaCl, contained laccase activity. SDS-PAGE gels showed a single 68-kDa band, indicating purification to homogeneity (Fig. 3A). In samples of the culture supernatant, precipitated crude extract, and purified enzyme, native gels showed a single broad activity band when developed with ABTS (Fig. 3B). This indicated that only one laccase band of 68 kDa was produced by \textit{P. sanguineus} CeIBMD001 under the growing conditions used in this study. To determine the number of isoforms present in this band, two-dimensional electrophoresis was carried out with purified enzyme. Two spots were observed after Coomassie staining of the 2D gels (not shown), both of which exhibited activity towards 2,6-DMP (Fig. 3C) or ABTS (not shown). This confirmed the existence of two isoforms with the same molecular mass but different pI values (7.0 and 7.08, Fig. 3C).

Enzymatic parameters (\(V_{\text{max}}\), \(K_m\), and \(k_{\text{cat}}\)) of the purified laccase were determined for the substrates ABTS, \(o\)-dianisidine, syringaldazine, and guaiacol (Table 1). Curves generated from plots of the reaction rate vs. substrate concentration for the different substrates demonstrated that the enzyme follows classical Michaelis-Menten kinetics (data not shown).

Interestingly, in contrast to other reports, our enzyme showed the highest affinity for \(o\)-dianisidine and the lowest for guaiacol. Similarly, specific activity of the purified laccase towards ABTS was 60.32 U/mg, in contrast to 20 U/mg reported by Garcia et al. [12]. Laccase retained high levels of activity in thermostability assays, with 80% and 40% of its original activity still present after incubation at 50°C and 60°C for 3 h, respectively; however, at 70°C activity was practically lost after one hour of incubation (retaining only 3.7%, Fig. 4A). Inactivation constants and \(t_{1/2}\) values measured at different temperatures are listed in Table 2. Long-term incubation at 40°C, nevertheless, showed that inactivation followed first-order decay kinetics, with 50% residual activity after 48 h and 10% after 5 days of incubation (Fig. 4B).

### Table 1. Kinetic parameters of the purified \textit{Pycnoporus sanguineus} laccase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m) (μM)</th>
<th>(V_{\text{max}}) (μmol ml(^{-1})min(^{-1}))</th>
<th>(k_{\text{cat}}) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS(^a)</td>
<td>238.55</td>
<td>4.36</td>
<td>94.176</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>693.08</td>
<td>0.479</td>
<td>10.361</td>
</tr>
<tr>
<td>o-Dianisidine</td>
<td>42.73</td>
<td>0.664</td>
<td>14.342</td>
</tr>
<tr>
<td>Syringaldazine</td>
<td>90</td>
<td>12.36</td>
<td>353.14</td>
</tr>
</tbody>
</table>

\(^a\)2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).

![Fig. 4](image-url) Thermal stability of the laccase produced by \textit{Pycnoporus sanguineus}. (A) Purified laccase was incubated at 40°C (closed circles), 50°C (open squares), 60°C (triangles), and 70°C (open circles) for the indicated time periods. (B) Laccase long-term stability at 40°C.
Discussion

Basidiomycetes (mainly isolated from temperate woods) are of interest because they are able to degrade xenobiotic compounds and thus may be applicable in the bioremediation of polluted environments. However, oil pollution affects many tropical areas where these fungi are not able to grow due to the high temperatures characteristic of these environments. It is therefore important to isolate native strains that can cope with these environments to be potentially used in bioremediation. We isolated a thermotolerant and halotolerant strain of \( P. \) sanguineus from an oil-polluted site in a tropical location. This strain grow at the elevated temperatures (47°C) and salt concentrations (500 mM) frequently present in tropical coastal areas, in contrast to other traditionally studied fungi such as \( B. \) adusta. In fact, the latter dies after 9 days of incubation at 37°C, a common temperature in the tropics. High concentrations of salt were also better tolerated by our isolate, an important feature for bioremediation in coastal areas. \( B. \) adusta was originally isolated, from among other fungi, as the strain best able to degrade PAHs [10], and it is currently widely used in bioremediation [18,21,22]. However, in high concentrations of oil \( P. \) sanguineus grew better than \( B. \) adusta. This suggests that our strain is at least as good as \( B. \) adusta for tolerating high concentrations of oil. It has the addition-al advantage of higher growth rates and greater resistance to the conditions common in oil spills in tropical coastal areas. This result is important given the potential presence of hydrolytic enzymes in species resistant to the toxic compounds found in oil. These observations highlight the importance of resistance to extreme conditions as a central attribute for potential bioremediation of tropical oil-polluted environments.

Several \( P. \) sanguineus strains and their laccases have been studied by other investigators. Garcia et al. [12] purified a 68- to 69-kDa laccase produced by a \( P. \) sanguineus strain. Even though the molecular mass of that laccase and the laccase studied here are similar, the two enzymes have different substrate affinities. Whereas syringaldazine is the best substrate for the laccase described by Garcia et al., the enzyme produced by our strain has a greater affinity for o-dianisidine. Additionally, the affinity of the laccase reported by Garcia et al., for ABTS and guaiacol is higher than that of our enzyme, by two- and four-fold respectively, indicating that the two enzymes are probably different isoforms. Litthauer et al. [17] purified a laccase of 58 kDa from a different strain of the same species. The kinetic parameters of that enzyme are different from those of other \( P. \) sanguineus laccases, including the enzyme described herein. Another difference is in the efficiency parameters (\( V_{\text{max}} \) and \( k_{\text{cat}} \)) of the various enzymes. Overall, our results and those previously reported provide evidence of the important variety of laccases produced by \( P. \) sanguineus strains, all of which may well have specialized niches of application. The molecular source of these differences remains to be determined. Our enzyme is composed of two isoforms with the same molecular mass but slightly different isoelectric points. Experiments are underway to determine whether these two isoforms are the products of one or more genes. It is worth noting that the pl of the native isoform is more basic than the pl values of other reported laccases, although the pl can vary widely, from 2.9 to 6.9 [1]. In this regard, the enzyme is similar to the \( Pleurotus \) laccases previously described [1].

The molecular mass, \( K_{\text{m}} \) and \( V_{\text{max}} \) for the enzyme characterized in this study fall within the range of previously reported laccases. Fungal laccases are known to possess very broad substrate affinities, and \( P. \) sanguineus CelBMD001 laccase is no exception. In general, laccases show greater affinity for ABTS than for other substrates, but our enzyme had a greater affinity for o-dianisidine. In accordance with other reports, it showed lesser affinity towards guaiacol [1,11]. Together, these results indicate that different strains can produce different laccases, each with its own unique features. The induction of different isoforms could be the result of the specific culture conditions and/or the genetic make-up of each strain. The culture medium used in this work contained high concentrations of starch and other sugars not present in the media used in other studies [11,16] and may have induced the particular isoforms detected.

For industrial applications, enzyme stability is a desirable quality. Some fungal laccases are thermostable, although most of the white-rot fungi laccases are not stable above 50°C. The laccase purified in this study retained 10% activity even after 5 days incubation at 40°C. The \( t_{1/2} \) values described here were lower than those of the \( P. \) sanguineus laccase reported by Litthauer et al. [16], although they were sufficiently high for most typical applications of the enzyme.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>( k_d ) (h(^{-1}))</th>
<th>( t_{1/2} ) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>3.290</td>
<td>0.21</td>
</tr>
<tr>
<td>60</td>
<td>0.303</td>
<td>2.28</td>
</tr>
<tr>
<td>50</td>
<td>0.066</td>
<td>10.50</td>
</tr>
<tr>
<td>40</td>
<td>0.018</td>
<td>38.5</td>
</tr>
</tbody>
</table>

Table 2. Laccase inactivation constants at different temperatures

The molecular mass, \( K_{\text{m}} \) and \( V_{\text{max}} \) for the enzyme characterized in this study fall within the range of previously reported laccases. Fungal laccases are known to possess very broad substrate affinities, and \( P. \) sanguineus CelBMD001 laccase is no exception. In general, laccases show greater affinity for ABTS than for other substrates, but our enzyme had a greater affinity for o-dianisidine. In accordance with other reports, it showed lesser affinity towards guaiacol [1,11]. Together, these results indicate that different strains can produce different laccases, each with its own unique features. The induction of different isoforms could be the result of the specific culture conditions and/or the genetic make-up of each strain. The culture medium used in this work contained high concentrations of starch and other sugars not present in the media used in other studies [11,16] and may have induced the particular isoforms detected.

For industrial applications, enzyme stability is a desirable quality. Some fungal laccases are thermostable, although most of the white-rot fungi laccases are not stable above 50°C. The laccase purified in this study retained 10% activity even after 5 days incubation at 40°C. The \( t_{1/2} \) values described here were lower than those of the \( P. \) sanguineus laccase reported by Litthauer et al. [16], although they were sufficiently high for most typical applications of the enzyme.
Another example of the robustness of this enzyme is that it can be run in a gel in the presence of SDS, and after its isolation it remains active. Experiments are currently being done to better characterize this property. All these features are important if this laccase is to be used, for example, in the presence of detergents to decolorize textile dyes.

Acknowledgements. This work was funded by grants SEP-PROMEP UAEMORPTC-59 and UAEMORPTC-119. O.V.-V. received a fellowship from CONACYT (number 181671); C.M.-A. was supported with a postdoctoral fellowship from CONACYT (exp. no. 050272). Technical assistance by Vanessa Hernández is gratefully acknowledged.

References