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

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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):XXX-XXX]

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 [23,25]. *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-

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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 Litthauer 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 thermotolerant 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 thermotolerant 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% $CaCO_3$) containing 240 U penicillin /ml. After successive passages, axenic cultures were identified morphologically and microscopically [19]. Dried and cryopreserved cultures have been deposited in the fungal collection of the National Herbarium of Mexico (MEXU 25347) of the National Autonomous University of Mexico (UNAM).

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% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H2O$, 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.

Thermotolerance, 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 28ºC. For thermotolerance assays, 0.16-cm^2 squares from the starter culture were placed on a fresh plate and incubated for an additional 24 h at 28ºC before the cultures were transferred to the experimental conditions (28, 37, 42, 45, 47, and 50ºC). 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 28ºC (*B. adusta*) or 37ºC (*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 Eléctricas May/June, 1998; http://www.iie.org.mx/publica/bolmj98/ secmj98.htm] as a dense Mexican oil of 22 API (American Petroleum Institute) gravity degrees and an asphlatene 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 flakes (BF) medium (3% ground Kellogg's Bran Flakes, in 60 mM phosphate buffer pH 6 [26]) and incubated at 200 rpm and 28ºC 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 induction 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 ($\varepsilon_{436} = 2.9 \times 10^4\ M^{-1}\ cm^{-1}$). On the day of maximum activity, supernatants were prepapred by filtering the mycelium and then centrifuging the filtrate for 20 min at 5250 × *g*. Activity was also determined with guaiacol ($\varepsilon_{470} = 2.6 \times 10^4\ M^{-1}\ cm^{-1}$), syringaldazine ($\varepsilon_{525} = 6.5 \times 10^4\ M^{-1}\ cm^{-1}$), and *o*-dianisidine ($\varepsilon_{46} = 1.1 \times 10^4\ M^{-1}\ cm^{-1}$). 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 –20ºC 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 Trap 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 (Centricon, Millipore; 30-kDa exclusion).

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

Gel electrophoresis. Laccase purity was determined by Coomasie 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 Coomasie BlueW or for laccase activity.

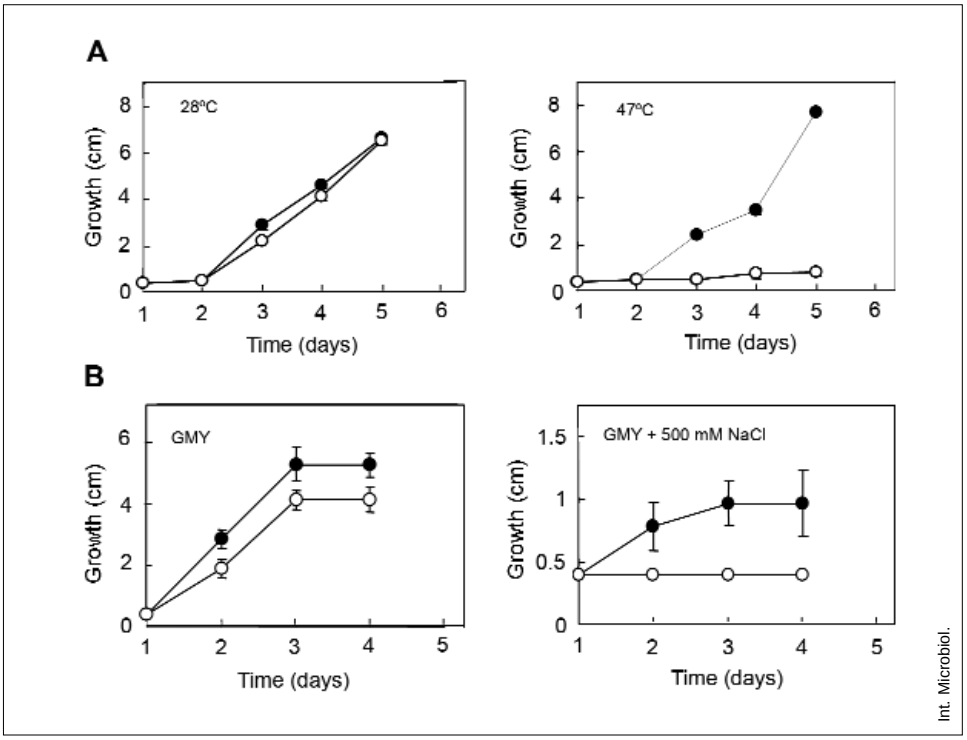

Fig. 1. (**A**) Growth at different temperatures of two basidiomycetes species: *Pycnoporus sanguineus* (closed circles) and *Bjerkandera adusta* (open circles). (**B**) Halotolerance of the same species growing without glucose-maltose yeast extract (GMY) or in the presence of 500 mM NaCl, at their temperature optima (28°C for *B. adusta*; 37°C, *P. sanguineus*). All experiments were performed in triplicate.

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 *(dAc/dt) = kdAc*, where Ac 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 $t_{1/2} = ln2/kd$.

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 (18º 06′ N, 94º 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 sequence 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 47ºC, in contrast to *B. adusta*. Furthermore, *B. adusta* died at 37ºC, since after 9 days at this temperature it was unable to resume growth when Petri dishes containing the cultures were transferred back to 28ºC. Growth of *P. sanguineus* was very slow at 50ºC, however, after 9 days under this condition, growth of the fungus recovered when it was transferred back to 28ºC (data not shown). The optimal growth temperature for *P. sanguineus* CeIBMD001 was 37ºC.

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

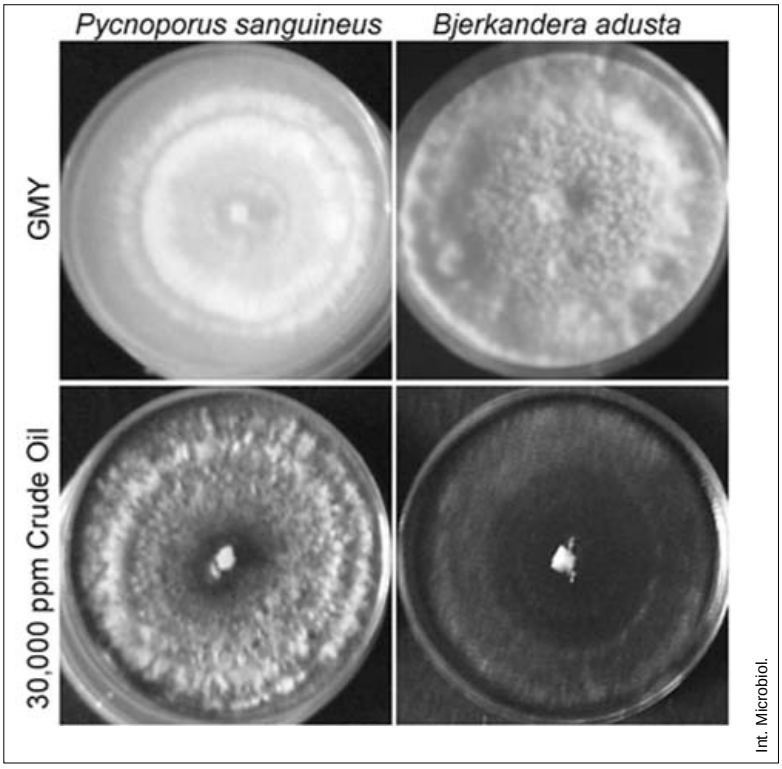

Fig. 2. Ability of *Pycnoporus sanguineus* (left panel) or *Bjerkandera adusta* (right panel) to grow in the presence of crude oil. Photographs show the representative results of three independent experiments.

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* CeIBMD001 is more capable of resisting harsher conditions than species from temperate climates.

Laccase purification and characterization. *Pycnoporus sanguineus* CeIBMD001 was grown on BF medium and extracellular laccase activity was measured for 14 days.

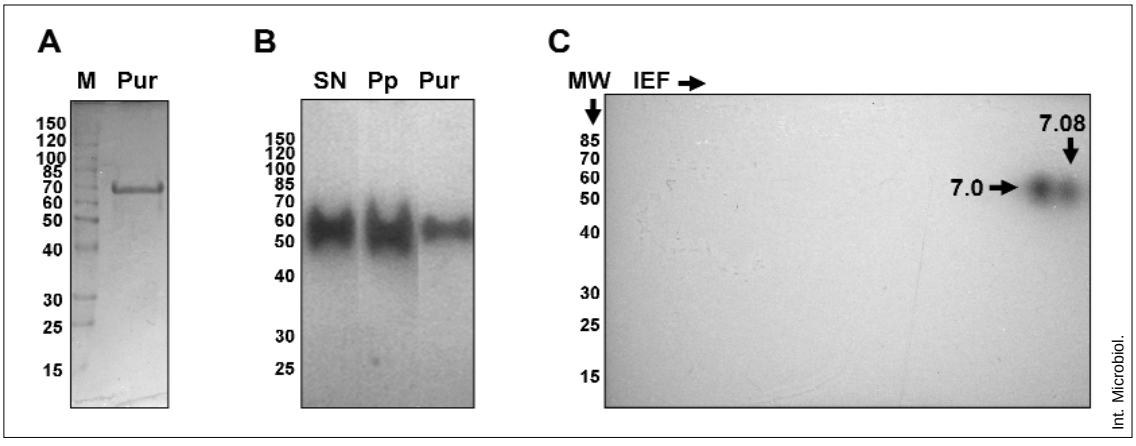

Fig. 3. Analysis of the laccase produced by *Pycnoporus sanguineus*. (**A**) One-dimensional gel electrophoresis of pure laccase. M, Molecular mass markers; Pur, fraction 24 of the High Trap Q elution. (**B**) ABTS activity staining at the different steps of the purification procedure. SN. Supernatant harvested after 7 days of culture; Pp, acetone precipitate; Pur, pure laccase. (**C**) Activity staining with 2,6-DMP following two-dimensional gel electrophoresis of the purified enzyme. Arrows indicate two laccase isoforms and their pI values.

Table 1. Kinetic parameters of the purified *Pycnoporus sanguineus* laccase

Substrate	K_m (μM)	V_{max} (μmol ml^{-1}min^{-1})	k_{cat} (s^{-1})
ABTS[a]	238.55	4.36	94.176
Guaiacol	693.08	0.479	10.361
o-Dianisidine	42.73	0.664	14.342
Syringaldazine	90	12.36	353.14

[a]2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).

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 *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 Coomasie 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_{max}, K_m, and k_{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. [13]. 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).

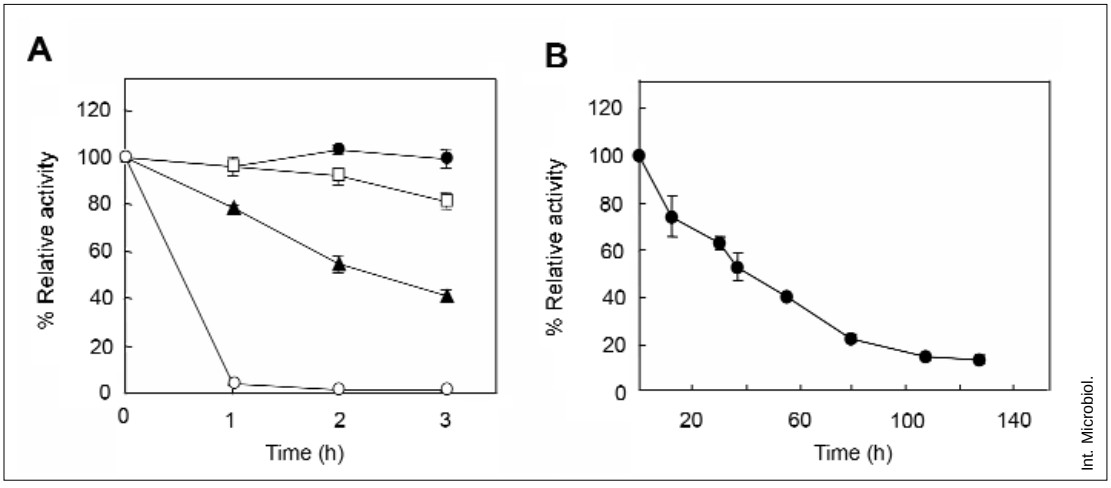

Fig. 4. Thermal stability of the laccase produced by *Pycnoporus sanguineus*. (**A**) Purified laccase was incubated at 40°C (closed circles), 50°C (open squares), 60°C (triangles), and 70°C (open circles) for the indicated time periods. (**B**) Laccase long-term stability at 40°C.

Table 2. Laccase inactivation constants at different temperatures

Temperature (ºC)	kd (h^{-1})	$t_{1/2}$ (h)
70	3.290	0.21
60	0.303	2.28
50	0.066	10.50
40	0.018	38.5

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 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 (47ºC) 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 37ºC, 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 [11], and it is currently widely used in bioremediation [19,23,24]. 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 additonal 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_{max} and k_{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 pI of the native isoform is more basic than the pI values of other reported laccases, although the pI 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_m, and V_{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* CeIBMD001 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 [1,10]. 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 [12,17] 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 50ºC. The laccase purified in this study retained 10% activity even after 5 days incubation at 40ºC. The $t_{1/2}$ values described here were lower than those of the *P. sanguineus* laccase reported by Litthauer et al. [17], 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.

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