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ORIGINAL ARTICLE

A Thermostable Metal-Tolerant Laccase with Bioremediation Potential from a Marine-Derived Fungus

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Abstract Laccase, an oxidoreductive enzyme, is important in bioremediation. Although marine fungi are potential sources of enzymes for industrial applications, they have been inadequately explored. The fungus MTCC 5159, isolated from decaying mangrove wood and identified as *Cerrena unicolor* based on the D1/D2 region of 28S and the 18S ribosomal DNA sequence, decolorized several synthetic dyes. Partially purified laccase reduced lignin content from sugarcane bagasse pulp by 36% within 24 h at 30°C. Laccase was the major lignin-degrading enzyme (~24,000 U L^{-1}) produced when grown in low-nitrogen medium with half-strength seawater. Three laccases, Lac I, Lac II, and Lac III, of differing molecular masses were produced. Each of these, further resolved into four isozymes by anion exchange chromatography. The N-terminal amino acid sequence of the major isozyme, Lac IId showed 70–85% homology to laccases from basidiomycetes. It contained an *N*-linked glycan content of 17%. The optimum pH and temperature for Lac IId were 3 and 70°C, respectively, the half-life at 70°C being 90 min. The enzyme was most stable at pH 9 and retained >60% of its activity up to 180 min at 50°C and 60°C. The enzyme was not inhibited by Pb, Fe, Ni, Li, Co, and Cd at 1 mmol. This is the first report on the characterization of thermostable metal-tolerant laccase from a marine-derived fungus with a potential for industrial application.

Keywords Marine-derived fungus · Basidiomycete · Laccase · Thermostable · Metal-tolerance · Bioremediation

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Introduction

Laccases (EC 1.10.3.2: benzenediol/oxygen oxidoreductase) are copper-containing lignin-degrading enzymes. They use molecular oxygen to oxidize a wide range of aromatic compounds besides lignin. Unlike the other lignin-degrading enzymes, lignin peroxidase and manganese-dependent peroxidase, laccase can be used in vitro for depolymerization of lignin or similarly structured aromatic compounds. Laccases do not have oxidation potentials as high as those of the peroxidases, but in the presence of a suitable redox mediator 2,2′-azino-bis (3-ethylthiazoline-6-sulfonate) (ABTS), their effective redox potential is increased and they can oxidize non-phenolic lignin model compounds. Basidiomycetous fungi are the major laccase producers (Baldrian 2006). Potential applications of laccases are in denim bleaching, decolorization and detoxification of dye-containing textile effluents, effluents containing lignin-related compounds, and biobleaching of pulp for paper industries (Baldrian 2006). Many such effluents also contain various inorganic chemicals such as sulfides, sulfates, chlorides, carbonates, peroxides, chlorine bleach compounds, and heavy metals. Heavy metals are known to be toxic to white-rot fungi (Baldrian and Gabriel 1997) and have a negative effect on the activity of ligninolytic enzymes in vitro (Baldrian et al. 1996). Therefore, new sources of

laccase through bio-prospecting with better properties such as high salt and heavy metal tolerance and thermostability for industrial applications are desired.

Obligately and facultatively, marine (marine-derived) fungi from lignocellulose substrates in the marine environment, particularly mangroves and sea grasses, are an important source of ligninocellulose-degrading enzymes (Raghukumar et al. 1994, 1999; Pointing and Hyde 2000; Bucher et al. 2004). They have been demonstrated to grow in the presence of high salt content (Raghukumar 2002, 2008; Raghukumar et al. 2008). A majority of fungi from mangroves were shown to produce enzymes such as cellulase, xylanase, and laccase in media prepared with seawater (Raghukumar et al. 1994; Pointing et al. 1998, 1999; Pointing and Hyde 2000). A marine-derived basidiomycete NIOCC #2a (MTCC 5159) isolated from mangrove wood produced laccase as the dominant lignin-degrading enzyme with negligible amounts of peroxidases in seawater medium (D'Souza et al. 2006). These authors reported enhanced production of laccase by this isolate in the presence of textile effluent (~85,000 U L^{-1}) and synthetic dyes. Various colored industrial effluents such as textile mill wastewater, molasses spent wash from alcohol distillery, and black liquor from paper and pulp industry were decolorized in the presence of live fungal culture and culture supernatant of MTCC 5159 (D'Souza et al. 2006; D'Souza-Ticlo et al. 2006). As these effluents are often contaminated with heavy metals and have temperatures above ambience, the heavy metal tolerance and thermostability of purified laccase from this marine-derived fungus MTCC 5159 was investigated. This enzyme was also further characterized and the results are presented here.

Materials and Methods

Organism, Culture Conditions, and Identification The basidiomycetous fungus NIOCC #2a was isolated from decaying mangrove wood, Chorao Island in Goa, India. It was maintained on Boyd and Kohlmeyer agar medium (Kohlmeyer and Kohlmeyer 1979) prepared with half-strength seawater (15–17 ppt) and was routinely checked for purity by light microscopy. The fungus was deposited in the Microbial Type Culture Collection (MTCC, Chandigarh, India) under the accession number MTCC 5159 under the Budapest treaty for patent culture deposition. As telomorphic stage of this fungus was not obtained in culture, it was identified by molecular methods. The fungus was identified using the D1/D2 region of 25-28S ribosomal DNA (rDNA; MIDI Labs, DE, USA). For ITS1-5.8S-ITS2 region, primers pITS1 (Fwd) (TCCGTAGGTGAACCTGCGG) and pITS4 (Rev) (TCCTCCGCTTATTGATATGC) were used (White et al. 1990). For 18S rDNA, two sets of universal primers were used to cover the entire length of the gene (White et al. 1990), the first set of primers being NS_1F (GTAGTCA TATGCTTGTCTC) and NS_4R (CTTCCGTCAATTCCTTT AAG) and the second set being NS_3F (GCAAGTCTGGTG CCAGCAGCC) and NS_8R (TCCGCAGGTTCACCTA CGGA). The amplification was performed in DNA Engine Thermal Cycler (BioRad, NSW, Australia). The polymerase chain reaction products were purified and sequencing was carried out by Microsynth Laboratories (Microsynth AG, Switzerland).

Dye Decolorization and Biobleaching Studies The laccase-hyperproducing NIOCC #2a was tested for its ability to decolorize synthetic dyes. These dyes, Congo Red, Methylene Blue (0.02 %), Trypan Blue, and Aniline Blue (0.04 %) were incorporated in low nitrogen (LN) agar medium. The plates were inoculated with plugs of this fungus and observed for decolorization up to 10 days.

Biobleaching of sugarcane bagasse pulp at 5% consistency was carried out with 100 U of laccase from this isolate at 30°C for 24 h. Reduction in lignin content was determined by estimating the kappa number in untreated and laccase-treated bagasse. The kappa number is an indication of the lignin content or bleachability of pulp. It is determined by the amount of 0.1 N $KMnO_4$ (in mL) which is absorbed by 1 g of oven-dried pulp under specific conditions and is then corrected to 50% consumption of $KMnO_4$. Its value indirectly indicates the lignin content or bleachability of pulp lignin, usually with yields of 70% or more. Kappa number was determined using $KMnO_4$-based method T236 (Anonymous 1988). Lignin content was obtained by multiplying the kappa number by a factor of 0.15. However the kappa number measurement is inflated by the presence of hexenuronic acids in the pulp. These compounds are formed from hemicelluloses during the chemical pulping process. Thus, the Kappa number measurement does not only represent the residual lignin in the pulp.

Culture Conditions LN medium (Tien and Kirk 1988) was modified to include glycine and fructose at 0.5% and 3.75% respectively, as the nitrogen and carbon sources instead of glucose and ammonium chloride. Among the trace metals, $CuSO_4$ was replaced with $CdCl_2$ at 0.08 g L^{-1}. The modified LN medium was prepared with half-strength seawater. The fungus was raised in the same medium for 6 days, homogenized in Omni Macro-homogenizer (model no. 17505, Marietta, GA, USA) for 5 s and the mycelial suspension was used as the inoculum. Erlenmeyer flasks (250 mL) containing 50 mL LN medium were inoculated with 5 mL mycelial inoculum (10%, *v*/*v*). The fungus was incubated at 30°C under static conditions. On day 6, $CuSO_4$ at 2 mM final concentration was added to the fungus under

aseptic conditions to stimulate laccase production. For large-scale cultivation, 3-L Haffkine flask with 1 L of LN medium was inoculated with 100 mL of mycelial suspension and incubated as described above.

Responses Measured The cultures were vacuum-filtered to remove the mycelium and the filtrate was stored at 4°C for enzyme estimation and purification. Responses were measured as fungal biomass, protein concentration, and laccase activity.

Fungal biomass was measured as dry weight. The fungus was filtered through pre-weighed Whatman no. 1 filter paper and dried at 60°C until a constant weight was obtained. The difference in weight was considered as the fungal dry weight and estimated in terms of grams per liter.

Protein concentration was determined using the Bradford reagent (Sigma, St. Louis, USA) and expressed as milligrams protein.

Laccase activity was estimated by measuring oxidation of 1 mM ABTS (2,2′-azino-bis-(3-ethylbenzothazoline-6-sulfonate) buffered with 0.2 M glycine–HCl buffer, pH 3, at 405 nm (Niku-Paavola et al. 1988). Activity was expressed in units defined as 1 μmol of product formed per minute per liter of culture supernatant (U L^{-1}).

All spectrophotometric measurements were carried out using UV–Vis 2450 spectrophotometer (Shimadzu, Japan). Values represent the mean of three measurements from two independent experiments.

Purification of Extracellular Laccase On day 12 of cultivation when laccase activity reached its maximum, the culture filtrate from Haffkine flask was collected after filtering the mycelium through GF/F and subsequently through 0.22-μm filter (Whatman Asia Pacific, Singapore). It was frozen at −20°C and all subsequent purification steps were carried out at 4°C. The precipitated exopolymeric substance produced by the fungus was removed from the thawed culture filtrate by centrifugation at 14,000 rpm for 15 min. The culture supernatant was then concentrated by ultrafiltration using YM3 membrane (Millipore, USA). The concentrate after filtering through 0.22-μm sterile filter was applied to High Load 16/60 Superdex 75 preparative grade column and eluted with 0.2 M Na acetate buffer (pH 4.5) containing 1 M KCl at a flow rate of 1 mL min^{-1} using a fast protein liquid chromatography system (Amersham Biosciences, Sweden). The molecular markers (Amersham Pharmacia, Sweden) used were bovine serum albumin (66 kDa), chicken egg albumin (45 kDa), carbonic anhydrase (29 kDa), and α-lactoalbumin (14.2 kDa). Out of the three major laccase peaks of differing molecular masses obtained during this size exclusion chromatographic step, the peak showing maximum absorbance at 280 nm (molecular mass ~56 kDa) and laccase activity was collected. It was concentrated using Amicon Ultracentrifugal filter tubes with a 5-kDa cutoff (Millipore, MA, USA). The concentrate was then applied to Mono Q™ 10/100 GL (Amersham Biosciences) column of 10×100 mm size and eluted with Tris–HCl buffer (pH 8) containing 0.5 M KCl at a flow rate of 0.3 mL min^{-1}. Out of several laccase peaks obtained with differing surface charges, the peak showing maximum laccase activity and absorbance at 280 nm was concentrated as above. This laccase fraction was termed as Lac IId and was used for further characterization. Purification and yield of laccase was carried out in three independent experiments.

Homogeneity of Lac IId was confirmed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) and 2D PAGE.

Determination of N-terminal and Internal Peptide Sequence of Lac IId For N-terminal amino acid (AA) sequence determination, Lac IId was electroblotted from an SDS-PAGE gel onto a polyvinylidene fluoride membrane (BioTrace, Pall Pharmalab Filtration, India) using mini trans-blot electrophoretic transfer cell (Bio-Rad, CA, USA). The AA sequence was then determined using an automated protein sequencing system (Procise HT-491, Applied Biosystems, CA, USA) at National Institute of Immunology, New Delhi, India.

For determination of the internal peptide sequence, the protein stained Lac IId from 2D-PAGE was digested in-gel by trypsin; the peptides were extracted by sonication and spotted on the α-cyano-4-hydroxycinnamic acid matrix for a matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) analysis. The sequencing was carried out at the Proteomics Facility, The Centre for Genomic Applications (TCGA), New Delhi using MALDI-TOF (Ultra Flex, Germany).

Determination of the Glycosylation Content of Lac IId For estimating the *N*-linked glycan content, 0.6 μg Lac IId was treated with 60 mU of endoglycosidase H (endo H, from *Streptococcus plicatus*, Sigma, MA, USA) overnight at 37°C. A non-denaturing 12% SDS-PAGE of the Endo H-treated and untreated Lac IId was then carried out. The gel was silver-stained for protein and with Schiff's reagent (Sigma) for glycoprotein (Coll et al. 1993; Xiao et al. 2003).

Isoelectric focusing, 2D PAGE, and Activity Staining Isozyme pattern of laccase in concentrated culture filtrate was determined by non-denaturing isoelectric focusing (IEF) with IEF strips of pH gradient 3 to 10 and 4 to 7 in IEF Cell (BioRad, CA, USA). The gel was stained for activity with guaiacol (5%) dissolved in citrate phosphate buffer (pH 6). For Lac IId, denaturing IEF was performed with pH gradient of 4 to 7 and p*I* was determined by silver staining (Heukeshoven and Dernick 1985).

For activity staining of laccase, non-denaturing SDS-PAGE (12%) was carried out and the laccase bands were visualized after staining with guaiacol. For determining the molecular mass of Lac IId, standard protein molecular markers of the medium range 14.3–97.4 kDa (PMWM, Bangalore Genei, India) were used and visualized by silver staining. A 2D PAGE (Bio-Rad) was run for confirming the p*I* and molecular mass of Lac IId.

Determination of pH and Temperature Optima of Lac IId To determine the pH and temperature optima of Lac IId, activity was measured with 1 mM ABTS in 0.2 M glycine–HCl (pH 2.5, 3), 0.2 M sodium citrate phosphate buffer (pH 3–6), 0.2 M Tris–HCl (pH 7–9). Each of the above assays (at varying pH) was performed in triplicates at 10° intervals from 20°C to 90°C.

Determination of pH and Temperature Stability of Lac IId To determine the pH stability, Lac IId was pre-incubated in triplicates at pH 2.5–9 for 1 h at 30°C and the residual activity estimated with ABTS, pH 3, at 70°C (at its optimum pH and temperature). For testing temperature stability, Lac IId was incubated in Tris–HCl buffer, pH 9 (at its maximum stability), in triplicates for various incubation periods at 50°C, 60°C, and 70°C, and the residual activity was estimated similarly.

Determination of Enzyme Kinetics Thermal characteristics for enzymatic reactions are described in accordance with kinetic parameters such as activation energy (E_a) which expresses the temperature dependence of the k value as indicated in the Arrhenius relationship: $\ln k = -E_a/RT + \ln A$. Hence, $k = A\left(e^{-E_a/RT}\right)$. The temperature coefficient Q_{10} value is the change in the rate of a reaction that occurs with a 10°C change in temperature. Thus, Q_{10}= [rate at temperature T+10°C]/[rate at T]. The Q_{10} value can be related to the Arrhenius equation as, $Q_{10}=e^{10E_a/RT}$, where E_a is independent of temperature provided that the conditions are appropriate and Q_{10} is dependent on temperature.

The effect of temperature on Lac IId activity was determined in triplicates by the Q_{10} value (Nelson and Cox 2004). If the rate of reaction is completely temperature-independent, the resulting Q_{10} will be 1. If the reaction rate increases with increasing temperature, Q_{10} will be greater than 1. Thus, the more temperature-dependent a process is, the higher will be its Q_{10} value.

Energy of activation was calculated from the Q_{10} values (Nelson and Cox 2004). The K_m constant and V_{max} of Lac IId were determined from Lineweaver–Burke plot using the substrate ABTS and syringaldazine (at their optimum pH and temperature). From these results, K_{cat} and specificity constant of Lac IId with ABTS were derived (Das et al. 2001).

Substrate Specificity and Inhibitors of Lac IId Oxygen uptake during reaction of Lac IId with substrates, non-substrates, mediators, and inhibitors was measured using an oxygen electrode (Oxygraph, Hansatech, Norfolk, England) at 30°C using 1-mL reaction chamber with 0.2 M citrate phosphate buffer at pH 4. The reaction mixture for inhibition measurements contained 1 U of Lac IId and 1 mM ABTS. Oxygen consumption was monitored for 15 min. The rate of oxygen uptake was measured in terms of nanomoles O_2 per minute per unit Lac IId. The results are expressed in terms of percent relative activity, considering laccase activity solely in the presence of ABTS as 100%.

Effect of Metal Ions on Lac IId Activity Effect of various metal ions at 1 mmol on Lac IId activity was measured spectrophotometrically with 1 mM ABTS at its optimum pH (3) and temperature (70°C). The activity in the absence of metal ions was considered as 100%, and the activity in the presence of metal ion was expressed as percent relative activity.

Results

Dye Decolorization and Biobleaching The fungus NIOCC #2a showed very good decolorization of the dyes Congo Red, Trypan Blue, Methylene Blue, and Aniline Blue in plate assay (Fig. 1). Laccase from this fungus also reduced lignin content of sugarcane bagasse by 36% within 24 h at 30°C. Based on these preliminary results, NIOCC #2a was used for further detailed studies.

Identification and Growth Characteristics of the Fungus NIOCC #2a had 99% and 98% identity to *Cerrena unicolor* (accession no. AY850007) with the 18S and 25–28S rDNA region, respectively. The ITS1-5.8S-ITS2 rDNA of NIOCC #2a showed homology to two strains of *C. unicolor* (EF577058 and DQ056858), which were the closest taxonomically identified hits with 91% homology. However, NIOCC #2a showed greater homology to basidiomycetes such as some unidentified marine-sponge-derived fungi (EF029829, EF029823, and EF029817) and an endophyte (AY456192) with 99% identity. These unidentified basidiomycetes could possibly be marine-adapted forms of *C. unicolor*. Hence, the fungus NIOCC #2a has been tentatively identified as a *C. unicolor* strain. The sequences of the ITS1-5.8S-ITS2, D1/D2, and the 18S regions of the rDNA are deposited in NCBI database under accession numbers AY939879, AY939878, and EF059806, respectively. The fungus was deposited in MTCC Chandigarh, India under accession no. MTCC 5159. Henceforth, this fungus is referred as *C. unicolor* MTCC 5159.

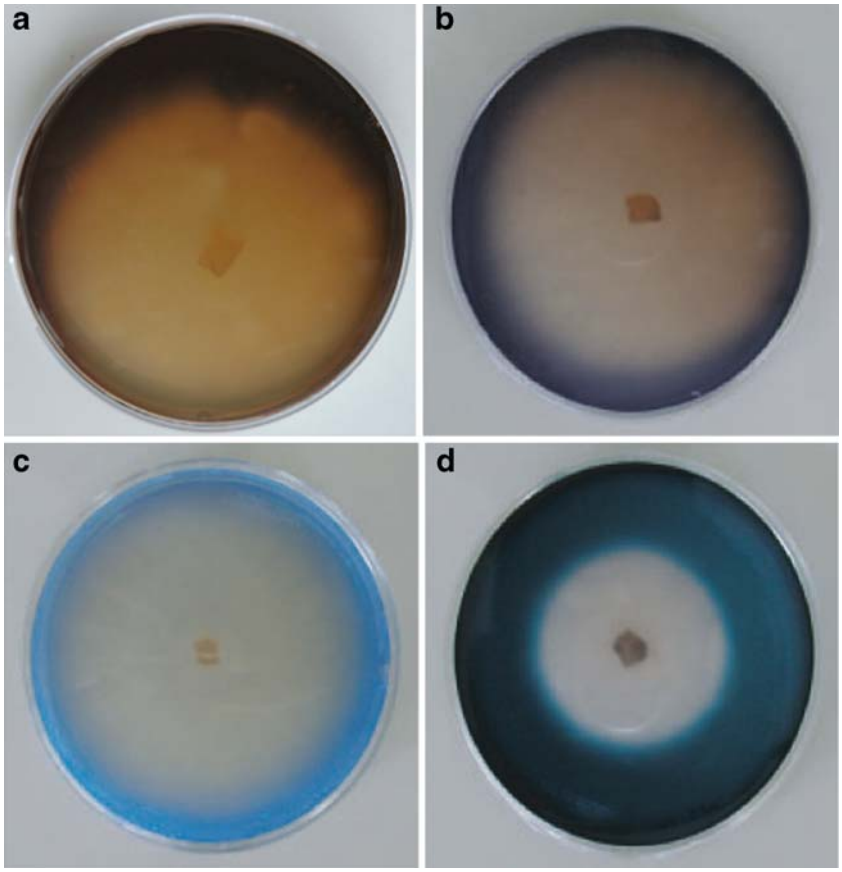

Fig. 1 Decolorization of Congo Red (**a**), Trypan Blue (**b**), Methylene Blue (**c**), and Aniline Blue (**d**) by MTCC 5159 in plate assay

As *Cerrena* is known to be a terrestrial fungus, its growth on mangrove wood could be a result of physiological adaptations, and therefore, its growth and laccase production in seawater medium were ascertained. *C. unicolor* MTCC 5159 showed growth and laccase production in LN medium prepared with seawater containing fructose and glycine as C and N source (Fig. 2). Laccase activity reached a maximum on day 12 (~12,600 U L^{-1}). Addition of catalase to the reaction did not affect rate of oxidation of ABTS, indicating that the entire oxidation of ABTS was contributed by laccase alone and not by any of the peroxidases. Lignin peroxidase and manganese-dependent peroxidase were negligible in this medium.

Purification of Laccase The fungus was harvested from 3-L Haffkine flasks on day 12 when laccase production reached its maximum, yielding 23,714 U L^{-1} with a specific activity of 330 U mg^{-1} protein. The concentrate when subjected to size exclusion chromatography (Superdex-75 column), yielded three major laccase peaks corresponding to molecular masses of 82, 56, and 45 kDa, and were termed Lac I, Lac II, and Lac III (Fig. 3a) respectively, among which Lac II showed maximum laccase activity and absorbance at 280 nm. By anion exchange chromatography (Mono Q column), Lac II further resolved into four major laccase peaks with differing surface charges (Fig. 3b). Lac I and III also resolved into four major laccase peaks, each with very low laccase activity (data not shown). This indicated that all the three molecular mass laccase isozymes also contained isozymes differing in their surface charges. Presence of

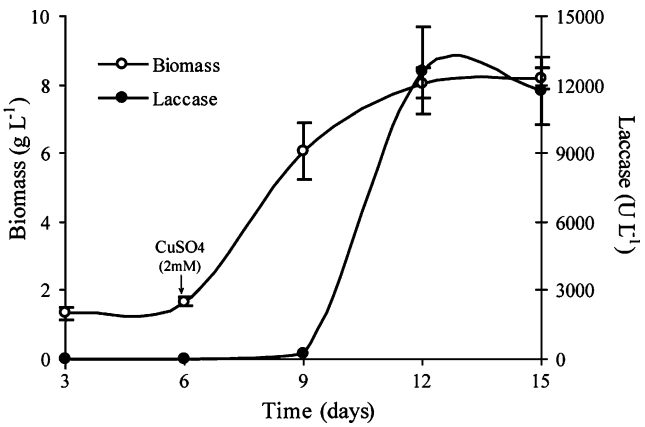

Fig. 2 Fungal biomass (*open circle*) and laccase production (*closed circle*) by *C. unicolor* MTCC 5159 in low nitrogen medium prepared in half-strength seawater containing fructose and glycine as C and N source, respectively. The fungus was maintained under shallow stationery conditions

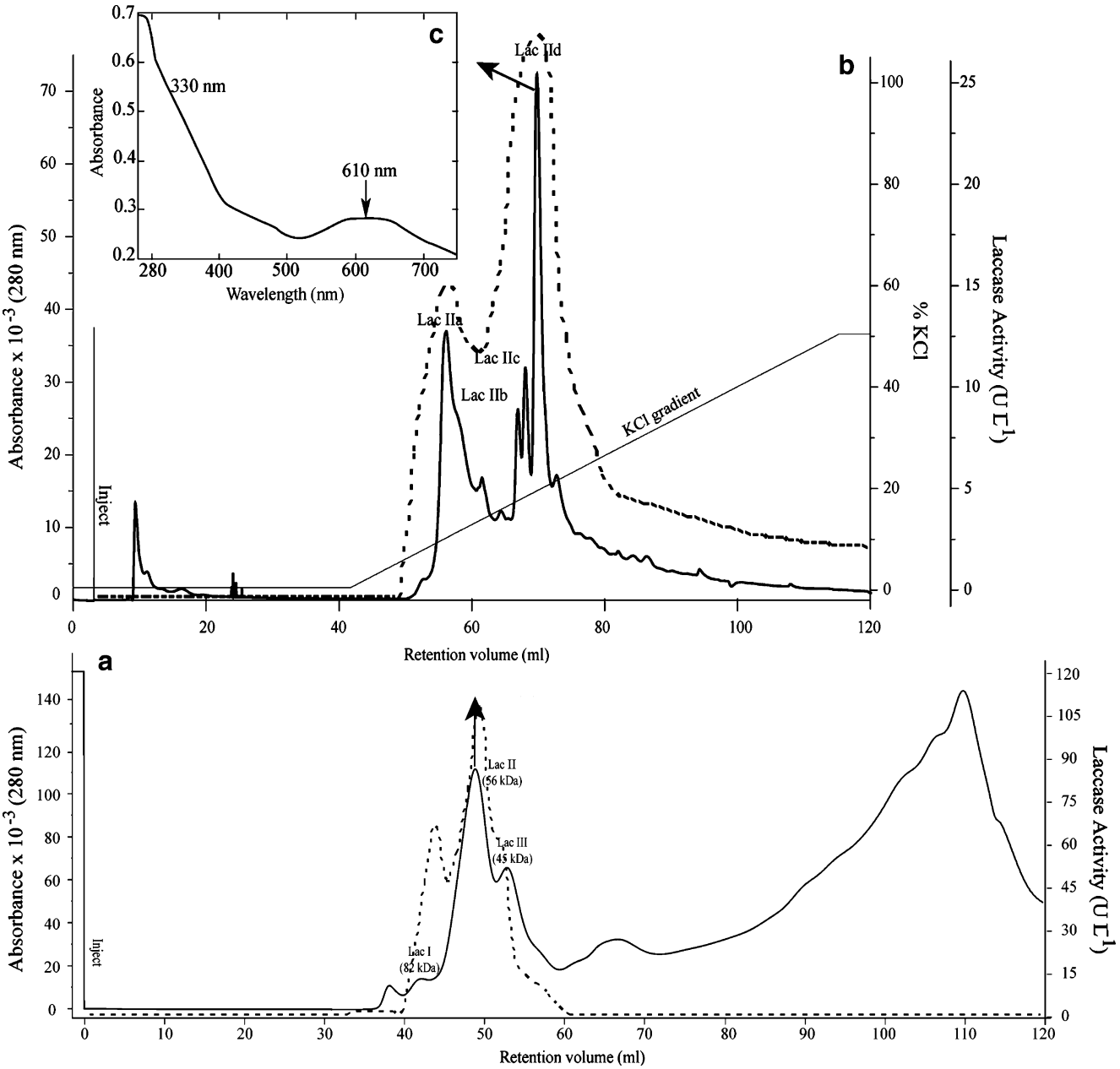

Fig. 3 **a** Size exclusion chromatography of the concentrated crude culture filtrate of a 12-day-old culture of *C. unicolor* MTCC 5159 (Superdex 75 column) showing three major laccase peaks (Lac I, Lac II, and Lac III, *broken line*). **b** Anion exchange chromatography of Lac II (Mono Q column) with KCl gradient of 0 to 0.5 M. Lac II resolved into four major laccase peaks termed Lac IIa, IIb, IIc, and IId (*broken line*). **c** A UV–visible spectrum of Lac IId showing a peak at 610 nm corresponding to type 1 Cu, typical of a blue laccase, and a shoulder at 330 nm corresponding to type 3 binuclear Cu pair

several isozymes (>20) with varying p*I*s in the concentrated crude culture filtrate was also confirmed by running a non-denaturing IEF (pH range 4–7) and subsequent staining for laccase activity (data not shown). From the anion exchange chromatography of Lac II, the peak which showed maximum laccase activity also corresponded to maximum absorbance at 280 nm (Fig. 3b). This was designated as Lac IId and selected for further characterization. Lac IId showed a molecular mass of 59 kDa and p*I* of 5.3 when analyzed by 2D PAGE. The UV–visible spectrum of Lac IId showed a peak at 610 nm, typical for the type 1 Cu(II) that is responsible for the blue color of the enzyme and a shoulder at 330 nm, which suggests the presence of the type 3 binuclear Cu(II) pair (Fig. 3c). Non-denaturing SDS-PAGE also confirmed the single band to be of 59 kDa (Fig. 4a, lane 4), which stained with guaiacol (Fig. 4b, lane 4) and had a p*I* of 5.3 (Fig. 4c) by IEF. A 33-fold purification was achieved with a final yield of 17% (Table 1).

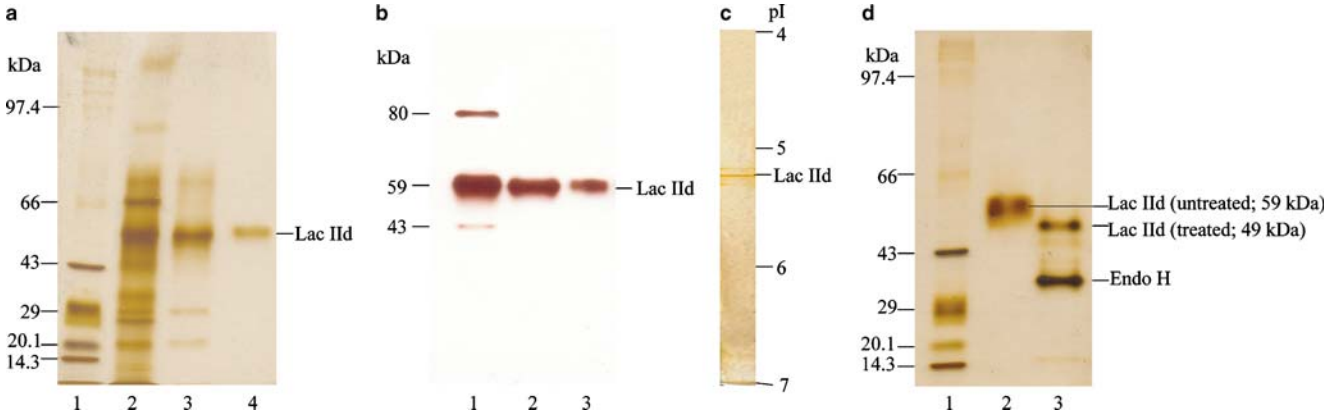

Fig. 4 **a** Non-denaturing SDS-PAGE (12%) of laccase from *C. unicolor* MTCC 5159. *Lane 1* PMWM marker; *lane 2* concentrated crude culture filtrate obtained by ultra filtration; *lane 3* Lac II (size exclusion chromatography); *lane 4* Lac IId (anion exchange chromatography). **b** Activity staining of laccase. *Lanes 1*, *2*, and *3* correspond to *lanes 2*, *3*, *4*, respectively, of **a**. **c** Silver staining of Lac IId on IEF strip showing a p*I* of 5.3. **d** Glycosylation status of Lac IId. *Lane 1* PMWM marker; *lane 2* Lac IId; *lane 3* Lac IId treated with endoglycosidase H

N-terminal and Internal Peptide Sequencing of Lac IId The N-terminal AA sequence of Lac IId showed 70–85% similarity with laccases of basidiomycetous fungi (Table 2). Maximum similarity was observed with laccases from the white-rot basidiomycetes, *Schizophyllum commune* and *Spongipellis* sp. The AA sequence was deposited under the accession number P85430 in the universal protein resource database, Uniprot (http://www.pir.uniprot.org).

From the MALDI-TOF analysis, the sequence of only one internal peptide of Lac IId could be positively identified (Table 2). This internal peptide sequence was also deposited in the universal protein resource database, Uniprot under the same accession number, P85430. A comparative study of various internal peptide sequences homologous to P85430 using the HSSP (Homology Derived Secondary Structure of Proteins, v. 1.1, 2001) was conducted after in silico digestion of the databases (protein, translated DNA, and expressed sequence tag) with trypsin. The internal peptide of P85430 matched with several known laccases. The maximum score of 51% at $p=0.05$, using the algorithm MASCOT (http://www.matrixscience.com/), was with a peptide "Q69FX1_9AGAR" present in Laccase 2 of *Volvariella volvacea* (AAR03581, from NCBI database). The predicted molecular mass and p*I* for AAR03581 was ~59 kDa and 5.4, respectively. This is very close to the experimentally determined molecular mass 59 kDa and p*I* 5.3 of Lac IId from this fungus.

Glycosylation Status of Lac IId On treatment with endoglycosidase H (Endo H), the molecular mass of Lac IId was reduced to 49 kDa (Fig. 4d, lane 3), revealing that it contained an *N*-linked glycan content of 17%. The untreated Lac IId stained for glycoprotein with Schiff's reagent, whereas the endo-H-treated sample did not.

Properties of Lac IId Lac IId showed optimum activity with ABTS at pH 3 and 70°C. With guaiacol and syringaldazine, it showed optimum activity at pH 6 (data not shown). It was most stable at pH 9 for 1 h at 30°C by retaining 100% of the activity (Fig. 5a). At pH 9, more than 60% of the activity was retained up to 180 min at both, 50°C and 60°C (Fig. 5b). About 50% residual activity was detected up to 90 min at 70°C (Fig. 5b). When the enzyme was incubated at its most stable pH of 9 in Tris–HCl buffer for 1 h at varying temperatures, it retained about 60% of its activity at 70°C (Fig. 5c). It retained 67% and 100% of its activity at 4°C and −20°C, respectively, for over 35 days. There was no loss in activity up to 1 year when stored at −20°C.

Table 1 Purification of extracellular laccase from *C. unicolor* MTCC 5159

Purification step	Volume (mL)	Total laccase (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification factor (fold)
Culture filtrate	1,000	23,714	71.9	330	100	1
Ultra filtration (5-kDa cutoff)	50	129,810	11.7	11,126	547	34
Size exclusion chromatography (Superdex 75)	2.5	3,039	0.92	3,315	2	10
Anion exchange chromatography (Mono-Q)	0.625	531	0.05	10,918	17	33

The results shown here are average of three independent experiments

Table 2 Alignment of the N-terminal and an internal peptide sequence of Lac IId (P85430) with other fungal laccases using T-Coffee version 5.0 (http://www.tcoffee.org)

N-terminal AA sequence

		Similarity (%)	E value
	1 10 20 30		
	|.....|.....|.....|		
Cerrena unicolor MTCC 5159 (P85430)	GTGPVADLHIINKDLSPDGFQRPTVVAGGG	100	6e-19
Schizophyllum commune (BAA31217)	ALGPVGNLPIVNKEIAPDGFSRPTVLANGK	85	8e-06
Spongipellis FERMP 18171 (BAE96003)	AIGPVADLHIVDVSIAPDGFSRPAVLAGGT	85	5e-06
Panus rudis (AAW28932)	AIGPVTDLHIVNDNIAPDGFTRAAVLAGGT	81	8e-06
Rigidoporus microporus (AAO38869)	AIGPVADLHISNANISPDGFTRAAVLAGGS	81	6e-06
Volvariella volvacea (AAR03582)	AIGPVTELQIVNDEIAPDGFSRGSVLANGA	77	0.018
Trametes I-62 (AAQ12270)	AVGPVTDLTISNANVSPDGFQRAAVVANGG	75	3e-05
Ganoderma lucidum (AAR82930)	AIGPVANLTISDADIAPDGFTRAAVVVNGV	70	0.25
	. *** :* * : .::**** * :*:..*		

Internal peptide

		Signal peptide (AA)	Start Position (AA) including signal peptide	Similarity (%)	E value
	1 5 10 15				
	|...|... .|...|..				
Cerrena unicolor MTCC 5159 (P85430)	RDVVSIGR--AGDNVTIRF	–	–	100	--
Volvariella volvacea (AAR03581)	RDVVSIGR--AGDNVTIRF	1-22	471	100	3e-07
Rigidoporus microporus (AAO38869)	RDVVSIGN--AGDNVTIRF	1-21	444	94	2e-05
Flammulina velutipes (BAE91880)	RDVVSIGA--AGDNVTIRF	--	423	94	2e-05
Laccaria bicolor (XP 001886681)	RDVVSIGG--AGDNVTIRF	1-17	439	94	4e-05
Pholiota nameko (ABR24264)	RDVVSIGG--AGDNVTIRF	1-18	444	94	4e-05
Panus rudis (AAW28932)	RDVVSTGT--AGDNVTIRF	1-21	445	88	3e-04
Trametes sp I-62 (AAQ12270)	RDVVSTGTPAAGDNVTIRF	1-25	448	--	0.008
Ganoderma lucidum (AAG17009)	RDVVSTGTPAAGDNVTIRF	1-21	444	--	0.008
	***** * *********				

The Q_{10} values of Lac IId which were >1 at its optimum pH 3 did not change drastically between 20°C and 70°C. In contrast, the Q_{10} values for temperatures above 70°C were <1. The energy of activation derived from Q_{10} values between 60°C and 70°C at this pH was 2.5 kJ mol^{-1}, whereas from the Arrhenius plot, it was 8.15 kJ mol^{-1}.

The K_m, V_{max}, and K_{cat} of Lac IId were compared using ABTS and syringaldazine; maximum specificity constant (K_{cat}/K_m) of 120 min^{-1} μM^{-1} was observed with ABTS at 70°C and pH 3 (Table 3). The kinetic parameters of Lac IId suggest that it has a higher affinity towards ABTS at 70°C than at 30°C. The K_m value suggests that at 30°C, syringaldazine is a better substrate than ABTS.

Substrate Specificity and Inhibitors of Lac IId The substrate specificity of Lac IId was measured by O_2 uptake (nanomoles per minute per unit Lac IId) in the presence of various phenolic compounds (Table 4). Among the substrates, ABTS was found to be the best followed by ferulic acid and guaiacol. Tyrosine, vanillic acid, 2,5-dimethyl aniline, *p*-anisidine, and violuric acid were not oxidized by Lac IId alone. However, in the presence of ABTS, most of the compounds, which previously did not get oxidized (with the exception of vanillic acid and tyrosine; Table 4), were oxidized. This indicates the mediating role of ABTS in the oxidation of these compounds when exposed to Lac IId. When ABTS was used as substrate, four of the known mediators were effective in increasing the laccase activity, confirming their role as mediators for Lac IId, whereas 4-hydroxy-2,2,6,6-tetra-methylpiperidin-1-oxyl did not elucidate any response and 1-hydroxybenzotriazole was antagonistic.

Among the various inhibitors tested for Lac IId, most of its activity was inhibited in the presence of sodium azide, SDS, and mercaptoethanol (Table 5). It was not inhibited by 4-hexyl resorcinol, a differential inhibitor of tyrosinase, a polyphenol oxidase very similar to laccase. The enzyme was not inhibited by L-cysteine and dithiothreitol.

Effect of Metal Ions on Lac IId Approximately 56% and 48% of Lac IId activity was inhibited in the presence of Cr and W, whereas in the presence of Sn, Ag, and Hg, the inhibition was only ~32–37% (Table 5). The other metal ions did not show significant inhibition.

Discussion

Obligate marine fungi grow and sporulate exclusively in the sea; on the other hand, facultative marine fungi or marine-derived fungi are capable of growth in the sea, having

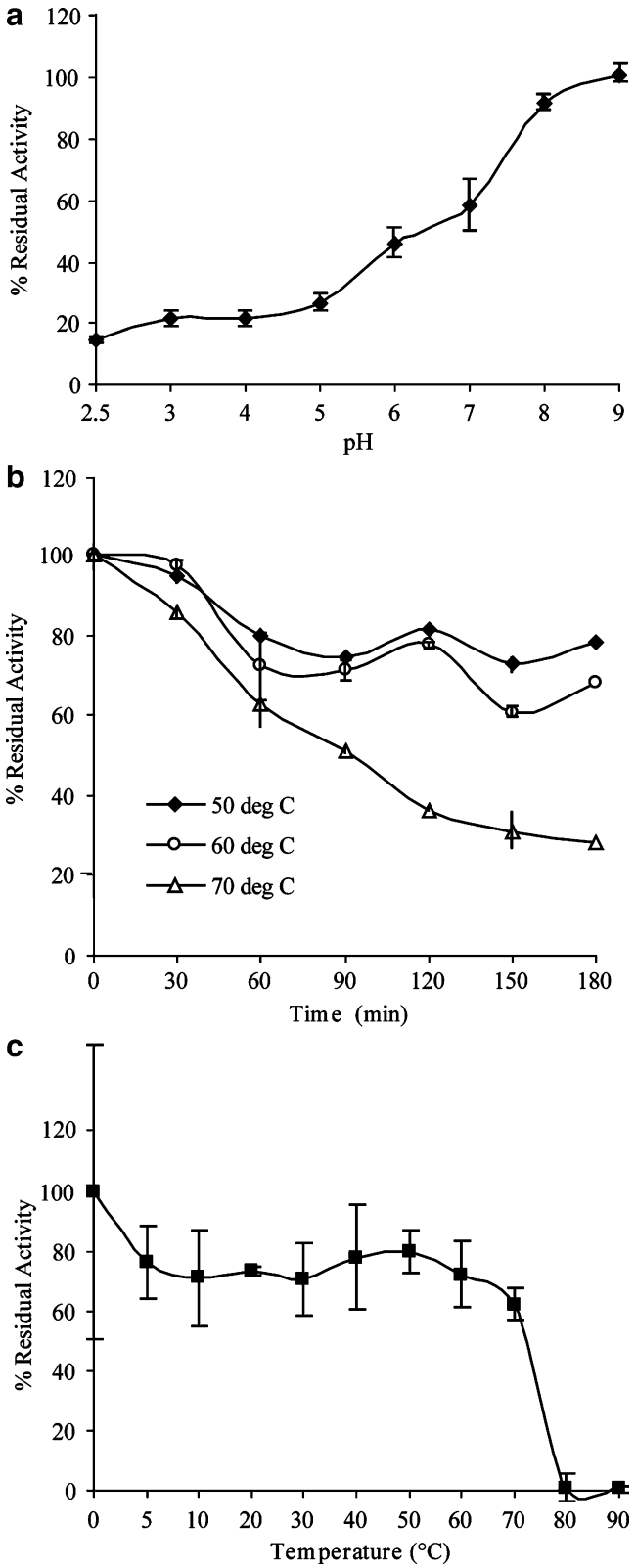

Fig. 5 Properties of Lac IId. **a** pH stability determined after incubation for 1 h at 30°C. **b** Thermostability at 50°C, 60°C, and 70°C at optimum pH stability (9). **c** Thermostability after incubation at various temperatures for 1 h at optimum pH (9) stability

become adapted to this environment. Several such fungi have been isolated from marine habitats and are reported to produce novel secondary metabolites and enzymes not seen in their terrestrial counterparts (Jensen and Fenical 2000; Raghukumar 2008). The present isolate MTCC 5159 appears to be one such highly potent marine-derived fungus. Although the isolate did not show any morphological features by which it could be identified, the 18S rDNA sequence identified it to be the basidiomycetous fungus *C. unicolor* (99% identity). It showed only 91% identity to *C. unicolor* in its ITS rDNA sequences but showed 99% identity to basidiomycetes associated with marine sponges, thus confirming its marine origin.

Although *C. unicolor* is a terrestrial fungus, the isolate MTCC 5159 obtained from mangroves appears to be a marine-adapted cryptic strain of the terrestrial species. This was evident by its growth and laccase production in media containing seawater (see Fig. 1). It could also decolorize dyes and colored effluents in seawater medium (D'Souza et al. 2006). The purified enzyme, Lac IId, was not inhibited in the presence of 1 mmol NaCl (see Table 5), and in the presence of half-strength seawater, it retained 75% of its activity (data not shown). Isoelectric focusing of the partially purified culture supernatant of this isolate grown in distilled water showed the major laccase isozymes around p*I* 4, whereas when grown in the medium containing full-strength seawater, these were around p*I* 7 (data not shown).

This isolate produced a titer of ~23,700 U L^{-1} laccase with a specific activity of 330 U mg^{-1} protein in low nitrogen medium prepared with seawater. In the presence of textile mill effluent (at 1%), MTCC 5159 produced a laccase titer of 85,829 U L^{-1} (D'Souza et al. 2006), whereas the terrestrial isolate of *C. unicolor* strain 137 was reported to produce only 18,700 U L^{-1} (Michniewiez et al. 2006). Several other terrestrial strains of *C. unicolor* were reported to produce much lower laccase titers (Gianfreda et al. 1998; Stepanova et al. 2003) than MTCC 5159. Although MTCC 5159 is known to produce LiP and MnP in low nitrogen medium, in the modified LN medium in the presence of $CuSO_4$ and $CdCl_2$, laccase was produced almost exclusively, making this medium ideal for the production, purification, and characterization of laccase. This strain produced a high titer of laccase in low as well as high nitrogen medium prepared with half-strength seawater.

The fungus produced three distinct laccases, Lac I, Lac II, and Lac III. Each one of these again yielded several isoforms. Presence of several guaiacol-stained laccase isozymes in the range of pH 4–7 during non-denaturing IEF of crude culture filtrate confirmed the existence of several isozymes differing in their p*I* in this isolate. From Lac II, the isoform which showed the maximum laccase activity, Lac IId has been purified and characterized. N-terminal amino acid sequencing, MALDI-

Table 3 Kinetic parameters of the Lac IId

Substrate[a]	K_m (μM)	V_{max} (μmol min^{-1})	K_{cat} (min^{-1})	Specificity constant (K_{cat}/K_m) (min^{-1} μM^{-1})
ABTS (70°C)	54.1	2.2	6,507	120
ABTS (30°C)	57.1	1.8	5,327	93
Syringaldazine (30°C)	19.2	0.7	2,112	110

Amount of Lac IId was held constant (0.02 μg protein) in all the assays carried out in triplicates

[a] Assay was carried out at optimum pH

TOF data, and 2D PAGE confirmed this to be a single isoform. The terrestrial isolate of *C. unicolor* strain 137 was reported to produce Lacc I and Lacc II, and on purification, together, they gave a yield of 22% (Michniewiez et al. 2006). The terrestrial *C. unicolor* strain T143 was reported to have one laccase which was purified to obtain 9.1% yield (Gianfreda et al. 1998). Kim et al. (2002) reported single laccase in the terrestrial *C. unicolor* strain CFC-120 which was purified to recover 23%. On the other hand, the marine-derived *C. unicolor* MTCC 5159 produced three major laccases differing in their molecular masses, Lac I, Lac II and Lac III, each of these resolving into four isoforms differing in their surface charges. A single isoform, Lac IId alone gave a yield of 17%. These results suggest physiological distinctiveness of this laccase-hyperproducing marine-derived isolate.

The Lac IId from MTCC 5159 has all the characteristics of a typical blue laccase: (1) blue color due to its absorbance at A_{610}, (2) a shoulder at A_{330} which is representative of type 3 binuclear copper pair, (3) N-terminal amino acid sequence

Table 4 Oxidation of various substrates, non-substrates, and mediators by Lac IId measured by oxygen uptake

	% Relative activity (SD)
Substrates (1 mM)	
ABTS	100 (27)
Ferulic acid	64 (9)
Guaiacol	53 (7)
Syringaldazine	42 (9)
L-Ascorbic acid	33 (13)
N,*N*-Dimethyl-*p*-phenylenediamine	21 (2)
Pyrocatechol	15 (3)
Indulin (0.25%)	15 (7)
p-Anisidine	0 (0)
2,5-Dimethyl aniline	0 (0)
Violuric acid	0 (0)
Vanillic acid	0 (0)
Tyrosine[a]	0 (0)
Non-substrates (1 mM)	
Control (only ABTS)	100 (11)
p-Anisidine	161 (12)
2,5-Dimethyl aniline	132 (16)
Violuric acid	122 (16)
L (−) Tyrosine	82 (7)
Vanillic acid	67 (8)
Mediators (1 mM)	
Control (only ABTS)	100 (11)
Hydroquinone	160 (21)
3,5-Dimethoxy-4-hydroxyacetophenone	128 (23)
Syringic acid	122 (10)
3,4-Dimethoxy benzyl alcohol	109 (13)
4-Hydroxy-2,2,6,6-tetra-methylpiperidin-1-oxyl	100 (15)
1-Hydroxybenzotriazole	86 (11)

Non-substrates and mediators were estimated in the presence of the substrate ABTS at 1 mM in triplicates [a] Tyrosine is not a substrate of laccase, it was included as a negative control

Table 5 Effect of inhibitors and metal ions on the activity of Lac IId in presence of 1 mM ABTS as substrate carried out in triplicates

	% Relative activity (SD)
Inhibitors (1 mM)	
Control (only ABTS)	100 (11)
DL-Dithiothreitol	160 (7)
4-Hexyl resorcinol	117 (10)
Cysteine	115 (9)
EDTA	104 (4)
Cysteine (0.1 mM)	104 (7)
o-Coumaric acid	101 (5)
DL-Dithiothreitol (10 mM)	80 (3)
8-Hydroxyquinoline	77 (10)
4-Nitrophenol	73 (7)
CTAB	69 (5)
Kojic acid	62 (3)
Tropolone	58 (9)
2-mercapto ethanol	23 (5)
SDS (1%)	23 (0)
Sodium azide (0.1 mM)	5 (3)
Metal ions (1 mmol)	
Control (only ABTS)	100 (9)
Mo^{+5}	103 (4)
Na^{+}	98 (5)
Ni^{+2}	97 (4)
Zn^{+2}	95 (1)
Cd^{+2}	95 (3)
Co^{+2}	94 (5)
Al^{+3}	94 (3)
Ca^{+2}	93 (2)
Cu^{+2}	93 (5)
Ba^{+2}	92 (2)
Mg^{+2}	90 (12)
As^{+3}	89 (7)
Pb^{+2}	84 (12)
Mn^{+2}	81 (6)
Li^{+2}	75 (3)
K^{+}	72 (2)
V^{+5}	72 (7)
Fe^{+3}	71 (2)

Inhibitors were measured by oxygen uptake. Metal ions were measured spectrophotometrically.

showing 70–85% homology to other basidiomycete laccases and the internal peptide showed 100% homology to a laccase from the basidiomycete, *V. volvacea*, (4) its inability to oxidize tyrosine is in concurrence with the fact that laccase is known for its inability to oxidize tyrosine, and (5) its non inhibition by 4-hexyl-resorcinol, a differential inhibitor of tyrosinase (Dawley and Flurkey 1993).

The Q_{10} value at its optimum pH (3.0) was >1 and did not change drastically between 20°C and 70°C. This indicates that up to 70°C, the activity of Lac IId was not negatively affected, which attests its thermostability. The optimum temperature for laccase activity in crude culture filtrate was 60°C (D'Souza et al. 2006), whereas for purified Lac IId, the optimum temperature was found to be 70°C. Laccase from terrestrial strains of *C. unicolor* showed optimum temperature of activity at 40°C and 60°C (Stepanova et al. 2003; Michniewiez et al. 2006). Lac IId of MTCC 5159 had a half-life of 90 min at 70°C. In contrast, Lacc I from terrestrial *C. unicolor* strain 137 lost its total activity in less than 10 min at 70°C, and Lacc II had a half-life of only 10 min at 70°C (Michniewiez et al. 2006). Energy of activation for Lac IId between 60°C and 70°C (2.5 kJ mol^{-1}) was much lower than at 20–30°C (13.4 kJ mol^{-1}), indicating that the enzyme is more efficient at higher temperatures. High turnover numbers for oxidation of ABTS by Lac IId at 70°C (K_{cat} and K_{cat}/K_m values) further indicates its thermostable character. High-temperature active laccase with thermostability at high temperature is desirable in biobleaching of pulp (Wong et al. 2000) and possibly also in treatment of colored industrial effluents (Asgher et al. 2008).

It is common for laccases from basidiomycetes to have optimum pH of activity in the acidic range and stability at neutral or alkaline pH (Xu et al. 1996). In concordance, Lac IId showed optimum activity at pH 3 and 70°C and was most stable at pH 9.

Lac IId of this isolate was not inhibited by several compounds that are generally inhibitory to laccase. *Pycnoporus cinnabarinus* laccase was totally inhibited by 1 mM L-cysteine and DTT (Eggert et al. 1996), whereas Lac IId from MTCC 5159 was neither inhibited in the presence of 0.1 nor 1 mM L-cysteine nor 1 mM DTT. Further, EDTA also did not inhibit its activity as was observed with the thermostable laccase from an unidentified basidiomycete (Jordaan et al. 2004). Sodium azide, an inhibitor of metalloenzymes (Heinzkill et al. 1998), maximally inhibited Lac IId. However, Lac IId was not affected by the addition of heavy metals such as Pb, Fe, Ni, Li, Co, and Cd at 1 mmol. Only Cr and W appeared to inhibit its activity by 48% and 56%, respectively. One of the problems of decolorization of colored industrial effluents is the presence of heavy metals. These are reported to have a negative effect on the action of lignin-degrading enzymes of white-rot fungi (Baldrian 2003).

Besides decolorization of dyes and effluents (D'Souza et al. 2006; D'Souza-Ticlo et al. 2006), the crude culture filtrate also reduced the lignin content in sugarcane bagasse. This process can be further improved by addition of natural mediators (Johannes and Majcherczyk 2000) to utilize this enzyme in biobleaching processes along with hemicellulases. Fungi producing lignin-degrading enzymes for

conversion of lignocellulose waste to create wealth in the form of biofuel are currently in great demand in the developing countries for meeting their growing energy demands (Howard et al. 2003). The basidiomycete MTCC 5159, with its high laccase titer and proven lignin-degrading ability, may find application in the pretreatment of lignocellulose waste. The tolerance of Lac IId to heavy metals, its high optimum temperature for activity and thermostability, as well as high laccase titer even in the presence of seawater makes the marine-derived *C. unicolor* MTCC 5159 a suitable candidate for application in treatment of effluents containing dyes, lignin-related compounds, chlorides, and sulfates.

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References

Annonymous (1988) Kappa number of pulp-T 236-cm-85. In: TAPPI test methods, vol 1. TAPPI, Atlanta, pp 1–3

Asgher M, Bhatti HN, Ashraf M, Legge RL (2008) Recent developments in biodegradation of industrial pollutants by white-rot fungi and their enzyme system. Biodegradation 19:771–783

Baldrian P (2003) Interactions of heavy metals with white-rot fungi. Enzyme Microb Technol 32:78–91

Baldrian P (2006) Fungal laccases: occurrence and properties. FEMS Microbial Rev 30:215–242

Baldrian P, Gabriel J (1997) Effect of heavy metals on the growth of selected wood-rotting basidiomycetes. Folia Microbiol 42:521–523

Baldrian P, Gabriel L, Nerud F (1996) Effect of cadmium on the ligninolytic activity of *Stereum hirsutum* and *Phanerochaete chrysosporium*. Folia Microbiol 41:363–367

Bucher VVC, Hyde KD, Pointing SB, Reddy CA (2004) Production of wood decay enzymes, mass loss and lignin solubilization in wood by marine ascomycetes and their anamorphs. Fungal Divers 15:1–14

Coll PM, Fernández-Abalos JM, Villanueva JR, Santamaria R, Pérez P (1993) Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971). Appl Environ Microbiol 59:2607–2613

Das N, Chakraborty TK, Mukherjee M (2001) Purification and characterization of a growth-regulating laccase from *Pleurotus florida*. J Basic Microbiol 41:261–267

Dawley RM, Flurkey WH (1993) Differentiation of tyrosinase and laccase using 4-hexyl-resorcinol, a tyrosinase inhibitor. Phytochemistry 33:281–284

D'Souza DT, Tiwari R, Sah AK, Raghukumar C (2006) Enhanced production of laccase by a marine fungus during treatment of colored effluents and synthetic dyes. Enzyme Microb Technol 38:504–511

D'Souza-Ticlo D, Verma AK, Mathew M, Raghukumar C (2006) Effect of nutrient nitrogen on laccase production, its isozyme pattern and effluent decolorization by the fungus NIOCC #2a isolated from mangrove wood. Indian J Mar Sci 35:364–372

Eggert C, Temp U, Eriksson K-EL (1996) The ligninolytic systems of the white-rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. Appl Environ Microbiol 62:1151–1158

Gianfreda L, Sannino F, Filazzola MT, Leonowicz A (1998) Catalytic behavior and detoxifying ability of a laccase from the fungal strain *Cerrena unicolor*. J Mol Catal B: Enzymatic 4:13–23

Heinzkill M, Bech L, Halkie T, Schneider P, Anke T (1998) Characterization of laccases and peroxidases from wood-rotting fungi (family Coprinaceae). Appl Environ Microbiol 64:601–606

Heukeshoven J, Dernick R (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis 6:103–112

Howard RL, Abotsi E, Jansen van Rensburg EL, Howard S (2003) Lignocellulose biotechnology: issues of bioconversion and enzyme production. African J Biotechnol 2:602–619

Jensen PR, Fenical W (2000) Marine microorganisms and drug discovery: current status and future potential. In: Fusetani N (ed) Drugs from the sea. Karger, Basel, pp 6–29

Jordaan J, Pletschke BI, Leukes WD (2004) Purification and partial characterization of a thermostable laccase from an unidentified basidiomycete. Enzyme Microb Technol 34:635–641

Johannes C, Majcherczyk A (2000) Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. Appl Environ Microbiol 66:524–528

Kim Y, Cho N-S Eom T-J, Shin W (2002) Purification and characterization of a laccase from *Cerrena unicolor* and its reactivity in lignin degradation. Bull Korean Chem Soc 23:985–989

Kohlmeyer J, Kohlmeyer E (1979) Marine mycology. The higher fungi, 1st edn. Academic, New York

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227:680–685

Michniewiez A, Ulrich R, Ledakowiez S, Hofrichter M (2006) The white-rot fungus *Cerrena unicolor* strain 137 produces two laccase isoforms with different physico-chemical and catalytic properties. Appl Microbiol Biotechnol 69:682–688

Nelson DL, Cox MM (2004) Lehninger principles of biochemistry, 4th edn. Freeman, New York

Niku-Paavola ML, Karhuner E, Salola P, Raunio V (1988) Lignolytic enzymes of the white-rot fungus *Phlebia radiata*. Biochem J 254:877–884

Pointing SB, Hyde KD (2000) Lignocellulose-degrading marine fungi. Biofouling 15:221–229

Pointing SB, Vrijmoed LLP, Jones EBG (1998) A qualitative assessment of lignocellulose degrading enzyme activity in marine fungi. Bot Mar 41:293–298

Pointing SB, Buswell JA, Jones EBG, Vrijmoed LLP (1999) Extracellular cellulolytic enzyme profiles of five lignicolous mangrove fungi. Mycol Res 103:696–700

Raghukumar C (2002) Bioremediation of colored pollutants by terrestrial versus facultative marine fungi. In: Hyde KD (ed) Fungi in marine environment. Fungal biodiversity research series 7. Fungal Diversity Press, Hong Kong, pp 317–344

Raghukumar C (2008) Marine fungal biotechnology: an ecological perspective. Fungal Divers 31:19–35

Raghukumar C, Raghukumar S, Chinnaraj A, Chandramohan D, D'Souza TM, Reddy CA (1994) Laccase and other lignocellulose modifying enzymes of marine fungi isolated from the coast of India. Bot Mar 37:515–523

Raghukumar C, D'Souza TM, Thorn RG, Reddy CA (1999) Lignin modifying enzymes of *Flavodon flavus*, a basidiomycete isolated from a coastal marine environment. Appl Environ Microbiol 65:2103–2111

Raghukumar C, D'Souza-Ticlo D, Verma AK (2008) Treatment of colored effluents with lignin-degrading enzymes: an emerging role of marine-derived fungi. Crit Rev Microbiol 34:189–206

Stepanova EV, Pegasova TV, Gavrilova VP, Landesman EO, Koroleva OV (2003) Extracellular laccases from *Cerrena unicolor* 059, *C. unicolor* 0784 and *Pleurotus ostreatus* 0432: a comparative assay. Appl Biochem Microbiol 39:375–381

Tien M, Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol 161:238–249

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis N, Gelfand D, Sninsky J, White TJ (eds) PCR-protocols and applications—a laboratory manual. Academic, New York, pp 315–322

Wong KKY, Richardson JD, Mansfield SD (2000) Enzymatic treatment of mechanical pulp fibres for improving papermaking properties. Biotechnol Prog 16:1025–1029

Xiao YZ, Tu XM, Wang J, Zhang M, Cheng Q, Zeng WY, Shi YY (2003) Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete *Trametes* sp. strain AH28-2. Appl Microbiol Biotechnol 60:700–707

Xu F, Shin W, Brown SH, Wahlleithner JA, Sundaram UM, Solomon EI (1996) A study of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity and stability. Biochim Biophys Acta 1292:303–311