

Chapter 3

Characterization
of Lac II_d,
a Laccase isozyme
from
Cerrena unicolor
MTCC 5159

ABSTRACT

The marine-derived, laccase-hyper-producing *Cerrena unicolor* MTCC 5159 was the source of laccase used in the present study. Among several methods tested to concentrate laccase, ultrafiltration using positive pressure was found to be most efficient. Partially purified laccase showed optimum activity at pH 3 and 60°C and contained a consortium of laccase isozymes.

Out of these, an isozyme, 'Lac IId' was purified with a final yield of 17 % and characterized in detail. It has a molecular mass of 59 kDa, a pI of 5.3 and 17 % *N*-linked glycosylation. The UV-visible spectrum showed a shoulder at 330 nm and peak at 610 nm which is characteristic of blue laccases. The N-terminal and an internal peptide sequence near the C-terminal are both deposited under the accession number P85430 in the 'Uniprot' database.

Lac IId showed optimum activity at pH 3 and 70°C. It has a half life of 90 min at 70°C and retained more than 60 % activity up to 180 min at 60°C at pH 9. Even after 1 year of storage at -20°C, no loss in activity was observed. Its energy of activation value of 2.5 kJ mol⁻¹ between 60° to 70°C at pH 3, attested to its thermostability. Lac IId could catalyze the oxidation of a wide range of substrates and its substrate range increased by the inclusion of mediators. Of all the inhibitors tested, only azide was able to effectively inhibit it up to 95 % of its original activity. Sodium chloride up to 0.3 M did not inhibit it above which, Lac IId was only reversibly inhibited. From the metal ions tested, Lac IId was maximally inhibited by chromium and retained 44 % of its original activity, whereas the other metals resulted in even lesser inhibition.

3.1 INTRODUCTION

Laccases (EC 1.10.3.2); benzenediol:oxygen oxidoreductase), are copper-containing lignin-degrading enzymes, often extracellular in nature. They use molecular oxygen to oxidize a wide range of aromatic compounds. Laccases are superior to other lignin-degrading enzymes, lignin-peroxidase and manganese-dependent peroxidase in that, the isolated enzyme can be used *in vitro* for depolymerization of lignin or lignin-related 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-ethyl benzothiazoline-6-sulphonic acid) (ABTS), their effective redox potential is increased i.e. they can oxidize nonphenolic lignin model compounds. Besides fungi, laccases are also found in plants, bacteria and insects. Among fungi, the white rot fungi are the major laccase producers (Baldrian, 2006).

Obligate and facultative (marine-derived) fungi from lignocellulose substrates in the marine environment, particularly mangroves and sea-grasses are an important source of lignin-degrading enzymes (LDEs) (Bucher *et al.*, 2004; Pointing & Hyde, 2000). A majority of fungi from mangroves were shown to produce cellulase, xylanase and laccase enzymes (Pointing *et al.*, 1998; Pointing & Hyde, 2000; Raghukumar *et al.*, 1994). Most of these studies have been qualitative or semi-quantitative. The LDEs from marine fungi have hardly been characterized in detail. The marine-derived fungus *Cerrena unicolor* MTCC 5159, a white-rot basidiomycete isolated from mangrove wood produced laccase as the dominant LDE in seawater medium (D'Souza *et al.*, 2006). The purification and characterization of laccase from this marine-derived basidiomycete is presented here.

A characteristic of laccase, besides being a blue copper protein is that it is glycosylated. The glycosylation of laccase has also served as a criterion in their classification. Laccases produced by plants often have 20 - 50 % glycosylation and are more involved in polymerization reactions. Whereas fungal laccases have 5 - 25 % glycosylation and are more involved in depolymerization reactions (Baldrian, 2006). Glycosylation of proteins is the enzymatic process that links saccharides (from an activated nucleotide sugar) to proteins, to produce glycoproteins. Three classes of glycans are produced which specifically attach to proteins: *N*-linked glycans attach to the amide

nitrogen of asparagine side chains, *O*-linked glycans attach to the hydroxy oxygen of serine and threonine side chains whereas glycosaminoglycans attach to the hydroxy oxygen of serine. *N*-linked glycosylation is important for the folding of some eukaryotic proteins. This process occurs in eukaryotes and widely in archaea but very rarely in bacteria.

Proteins can be glycosylated by both types of oligos at different sites on the protein. In laccases, the glycans are *N*-linked to the polypeptide chain (Brown *et al.*, 2002; Ko *et al.*, 2001; Saparrat *et al.*, 2002). Glycosylation serves various functions, for instance, some proteins do not fold correctly unless they are glycosylated first. Also, polysaccharides linked at the amide nitrogen of asparagine in the protein confer stability on some secreted glycoproteins. Glycosylation may not be a strict requirement for proper folding but the unglycosylated protein degrades quickly. It was proposed that in addition to the structural role, glycosylation can also participate in the protection of laccase from degradation by proteases (Yoshitake *et al.*, 1993), which are often present in the harsh extracellular environment into which laccases are secreted in.

In general, laccase can catalyze the oxidation of a wide array of substrates due its low specificity for the reducing substrate. The greater the range of substrates it can oxidize, greater is its commercial and biotechnological impact. The substrates range from metal complexes like ferrocyanide to phenolic and aromatic compounds to inorganic, organic and even biological compounds. The range of the substrates that can be oxidized by laccase is determined by the redox potential of the T1 Cu site. As long as the redox potential of a substrate is lower than that of the T1 Cu site, the laccase will be capable of catalyzing its oxidation (Gianfreda *et al.*, 1999). Also, crystallographic studies on the substrate-binding pocket of *Coprinus cinereus* laccase indicate that this binding-pocket is shallow and thus has limited steric hindrance on simple phenolic substrates (Ducros *et al.*, 1998; Gianfreda *et al.*, 1999; Hyde *et al.*, 2000; Xu, 1996) resulting in easy access of substrate to the active site. Physical methodology such as circular dichroism (CD) and electron paramagnetic resonance (EPR) have been used to study the metal centers of fungal laccases, showing that they are relatively stable compared to the secondary structure of the protein (Bonomo *et al.*, 2001). The secondary structure can undergo changes to alter substrate specificity. Numerous substrates have been used to study laccase activity. The use of syringaldazine or ABTS as a substrate can be flawed, since

both these substrates are also oxidized by manganese dependant peroxidase and lignin peroxidase which are often produced along with laccase. Special precautions involving removal of hydrogen peroxide is frequently omitted in work in which the presence of laccase is supposedly shown. Similarly, laccase and tyrosinase share a large number of substrates. To differentiate between laccase and peroxidase on the one hand and between laccase and tyrosinase on the other requires several proofs. One of the difficulties is the fact that the immediate product of laccase activity is a semi-quinone i.e. a free radical, which is extremely reactive and able to interact with numerous compounds which are present particularly in inadequately purified enzyme preparations. The complexity of such reactions has been shown by Ferrari *et al.* (1997). The only conclusive proof of laccase activity according to Mayer & Staples (2002) is that it is able to oxidize quinol as determined by oxygen uptake no matter what other substrates are attacked.

Tyrosinases, catechol oxidases and laccases were earlier summarized as phenoloxidases and share a common substrate range. This often leads to misidentification. There are structural differences between the two enzymes. Tyrosinases have a coupled binuclear copper complex at their active site i.e. only two T3 copper atoms are present and are best known for their polymerization reactions often leading to the formation of melanin. Laccase on the other hand, contains one T1 copper at the substrate binding site and a trinuclear copper center which contains one T2 and two T3 coppers at the co-substrate-binding site. Laccases are capable of both polymerization as well as depolymerization reactions, the direction of the reaction depends on laccase molecular structure and the type and quantity of substrate as well as product build up, in the reaction environment.

Although their end-products are often the same and both enzymes catalyze the four-electron reduction of O_2 to H_2O , the mechanisms by which they achieve their goal are different (Fig. 1). Tyrosinases (EC 1.14.18.1) comprise two reactions; mono- and di-phenolase activities i.e. hydroxylation followed by oxidation reactions. For the hydroxylation reaction, one atom of O_2 is incorporated into the aromatic ring of the monophenol, resulting in *o*-diphenol (mono-phenolase / cresolase activity). This *o*-diphenol is then converted into its benzoquinone by the oxidation reaction (diphenolase / catecholase activity). In this respect, tyrosinase is a 'mixed-function oxidase'. The oxidation step does not require the exclusive presence of tyrosinase, auto oxidation also

works. The catechol oxidases (EC 1.10.3.1) exert only the diphenolase activity but not the chemically fastidious hydroxylase step. Whereas Laccases (EC 1.10.3.2) have no hydroxylase activity but oxidize mono- and diphenols by the generation of radicals of the substrate they are attacking.

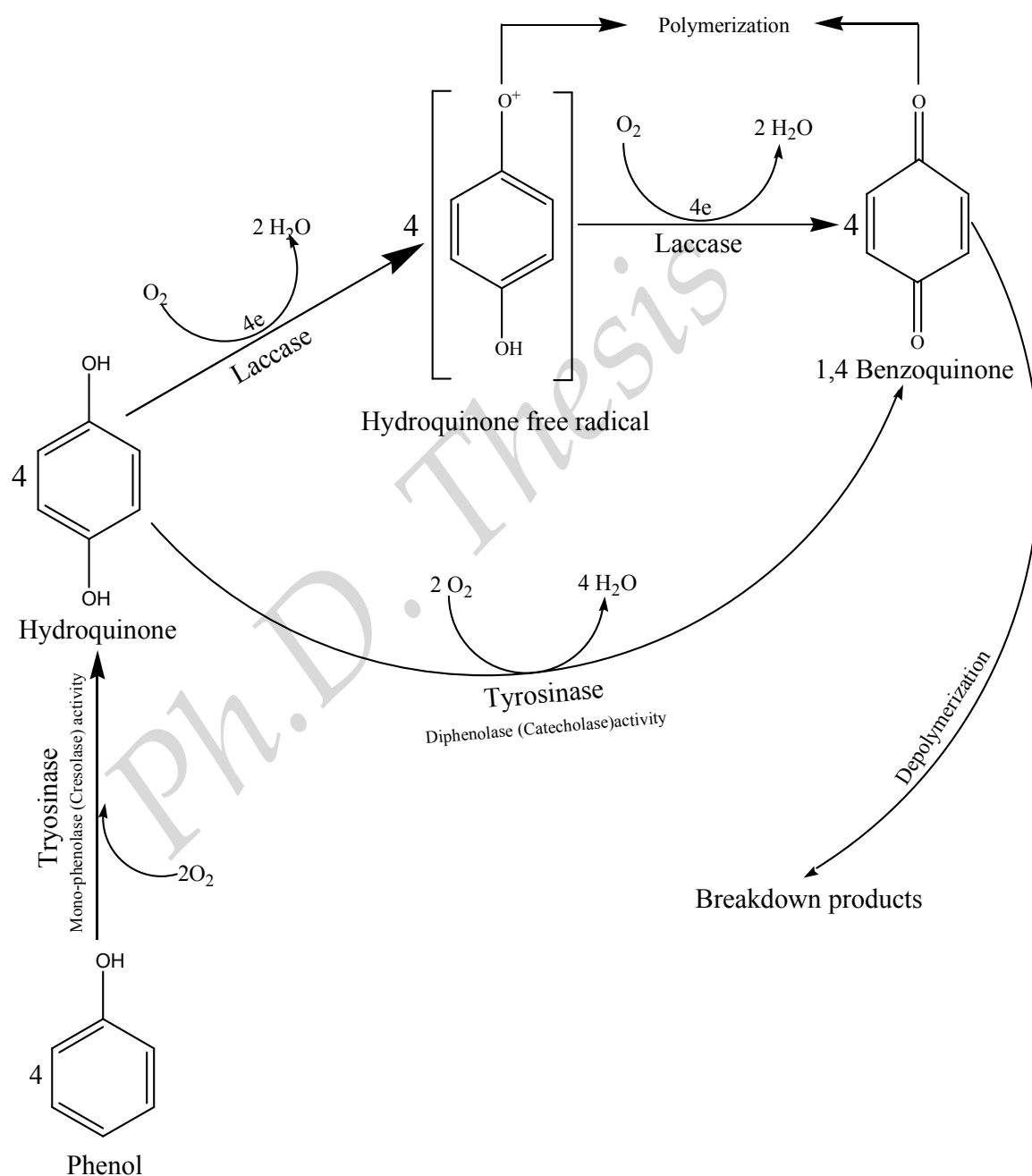


Fig. 1: Difference in mechanism of action between tyrosinase and laccase.

For tyrosinase the pH optima ranged from 3 (*Vanilla planifolia*) to 9.6 (*Pinus densiflora*), could even work at pH 11 (*Thermomicrobium roseum*). Whereas for laccase, the pH optima ranged from 2 (*Daedalea quercina*), to 8 (*Coriolus versicolor*) *Myrothecium verrucaria* laccase can work at even pH 11.5. On the whole tyrosinases often have the optimum working pH in the alkaline range whereas laccases in the acidic range.

Based on their catalytic properties, tyrosinases and laccases can be also differentiated. Cetyltrimmonium bromide (CTAB), an inhibitor of laccase does not have much effect on tyrosinase. The specificity of tyrosinases for substrates like tyrosine and selective inhibition by inhibitors such as carbon monoxide, 4-hexylresorcinol or salicylhydroxamic acid (Allen & Walker, 1988; Dawley & Flurkey, 1993; Petroski *et al.*, 1980) facilitate their differentiation from laccases when protein purification is not successful.

Presented below are a few techniques regularly used in purifying and characterizing laccases.

Size exclusion chromatography is a chromatographic method wherein particles like proteins are separated based on their size in more technical terms, their *hydrodynamic volume* (Lathe & Ruthven, 1956). It is known as 'gel filtration' when an aqueous solution is used as the mobile phase and polyacrylamide, dextran or agarose is used as the gel medium or the stationary phase. The underlying principle is that proteins of different sizes will elute (filter) through the stationary phase of a fixed pore size (cut off determined according to the protein to be purified) at different rates. The elution volume decreases roughly linearly with the logarithm of the molecular hydrodynamic volume (for globular proteins this is proportional to their molecular mass). The collected fractions are detected by spectroscopic techniques like ultraviolet (UV) absorbance, at 280 nm for protein.

The advantage of this method is that various solutions can be applied without interfering with the filtration process, while preserving the biological activity of the soluble proteins to be separated. This technique is generally combined with others that further separate molecules by other characteristics, such as affinity (lectins for glycoproteins such as laccase are used) and charge (Wilson & Walker, 2000).

Ion exchange chromatography relies on the interactions between the charges of the sample protein and the charge of the immobilized on the resin which is used for separation. It is of two types; *cation exchange chromatography*, where positively charged proteins bind to a negatively charged resin and *anion exchange chromatography* where the reverse occurs. Once the solutes are bound, the column is equilibrated with a low ionic strength starting buffer and the bound proteins are eluted off using a gradient of eluent buffer, with steadily increasing ionic strength. Alternatively a buffer with steadily increasing/ decreasing pH values may also be used as eluent in some cases (Wilson & Walker, 2000).

Two-Dimensional PolyAcrylamide Gel Electrophoresis (2-D PAGE) separates proteins according to charge (pI) by isoelectric focusing (IEF) in the first dimension and according to size (Mr) by SDS PAGE in the second dimension (O' Farrell, 1975).

In IEF, the protein is run on a pre-formed (by ampholytes) pH gradient. The protein stops moving in the pH gradient corresponding to its isoelectric point (pI) or Zwitter ionic state. Problems of pH gradient instability and reproducibility were resolved using Immobilized pH Gradients (IPG) (Bjellqvist *et al.*, 1982). This is based on the principle that the pH gradient is generated by a limited number (6 - 8) of well-defined chemicals (the 'Immobilines') which are co-polymerized with the acrylamide matrix. Thus, cathodic drift is eliminated, reproducibility enhanced and pattern matching and inter-laboratory comparisons simplified. The IPGs allows for the generation of pH gradients of any desired range (broad, narrow or ultra-narrow) between pH 3 and 12 and offers high sample loading capacity as well.

The second dimension (traditional SDS PAGE) can be run on horizontal or vertical systems (Görg *et al.*, 1998). In the vertical setup, the equilibrated IPG gel strips are placed on top of the vertical SDS gels and embedded in agarose. After termination of the second dimension run, the separated proteins in the gel are immobilized and interfering non-protein components removed by fixing. The separated polypeptides can be visualized by 'general protein' stains like Coomassie blue, silver, fluorescence or autoradiography, or by 'specific protein' stains such as Schiff staining for glycoprotein or immunochemical detection methods. Silver staining methods are about 10 - 100 times more sensitive than various Coomassie blue staining techniques. General protein stains

are carried out directly in the gel whereas immunochemical detection methods are usually performed after electrophoretic transfer 'blotting' of the separated polypeptides from the electrophoresis gel onto an immobilizing membrane (Kyhse-Andersen, 1984).

It is preferable to gain N-terminal or even internal amino acid sequence information of proteins separated by 2D-PAGE. The stained spots excised from the gel or the blotting membrane can be subjected directly or after cleavage, to amino acid sequencing, amino acid composition analysis, or mass spectrometry (Wilm *et al.*, 1996).

N-terminal sequencing is a chemical process based on the technique developed by Pehr Edman in the 1950's. The N-terminal amino acid (the end of the protein which is terminated by a free amine, -NH₂ group) is derivatized with phenylisothiocyanate (PITC). The derivatizing process results in a phenylthiohydantoin (PTH)-amino acid. This amino acid is then sequentially removed, while the rest of the peptide chain remains intact. Each derivatization process is a cycle. Each cycle removes a new amino acid. The amino acids are sequentially analyzed to give the amino acid sequence of the protein or peptide. These sequentially removed amino acid residues from the N-terminus of the protein are then identified by reversed phase HPLC. Pure proteins (> 90 %) usually generate easily interpreted data. Long sequences of 50 amino acids or more are possible with this technique.

Matrix-Assisted Laser Desorption / Ionization (MALDI) is a soft ionization technique used in mass spectrometry (Karas *et al.*, 1987). This allows the analysis of biomolecules such as proteins, peptides and sugars which tend to be fragile and fragment when ionized by more conventional ionization methods. In proteomics, MALDI is used for the identification of proteins isolated through 1D or 2D SDS PAGE. One method used is peptide mass fingerprinting by MALDI-MS, carried out by digests of the protein in question.

In MALDI, the ionization is triggered by normally a nitrogen laser. A matrix is used to protect the biomolecule from being destroyed by direct laser beam and to facilitate vaporization and ionization. The matrix consists of crystallized molecules, of which the three most commonly used are sinapinic acid (for glycoproteins and glycopeptides), α -cyano-4-hydroxycinnamic acid (HCCA) and 2,5-dihydroxybenzoic acid

(DHB). A solution of one of these molecules is made often in a mixture of highly purified water and an organic solvent such as acetonitrile or ethanol, trifluoroacetic acid may also be added. The matrix solution is mixed with the analyte (e.g. protein-sample). The organic solvent allows hydrophobic molecules to dissolve into the solution while the water allows for water-soluble (hydrophilic) molecules to do the same. This solution is spotted onto a MALDI plate. The solvents vaporize, leaving only the recrystallized matrix but now with analyte molecules spread throughout the crystals. The matrix and the analyte are co-crystallized in a MALDI spot. The laser is fired at the crystals in the MALDI spot. The matrix absorbs the laser energy and then transfers part of its charge to the protein thus ionizing it while still protecting it from the disruptive energy of the laser. Ions observed after this process consist of a neutral molecule [M] and an added or removed ion. Together, they form a quasimolecular ion, for example $[M+H]^+$ in the case of an added proton, $[M+Na]^+$ in the case of an added Na ion, or $[M-H]^-$ in the case of a removed proton.

The type of mass spectrometer most widely used with MALDI is the TOF MS (Time-Of-Flight Mass Spectrometer), mainly due to its large mass range. The TOF measurement procedure is also ideally suited to the MALDI ionization process since the pulsed laser takes individual 'shots' rather than working in continuous operation. Proteins / peptides can be sequenced using MALDI -TOF-MS with a ladder sequencing technique, which consists of either a time-dependent or concentration-dependent chemical degradation from either the N- or C-terminus of the protein / peptide into fragments, each of which differs by one amino acid residue. The mixture is mass analyzed in a single MALDI-TOF-MS experiment with mass differences between adjacent mass spectral peaks corresponding to a specific amino acid residue. This type of analysis determines the masses of a series of peptides / proteins that are present in a single MALDI sample. The order of occurrence in the mass spectrum defines the sequence of amino acids in the original protein / peptide. The sequence information for an unknown peptide or protein sequence is deduced from its metastable ion decay mass spectrum.

Protein identification by MALDI-TOF is carried out by digesting the protein with trypsin (cleaves at R-X and K-X except when X is Pro) and a peptide mass fingerprint produced by analyzing the digested protein with a MALDI-TOF MS generating monoisotopic masses of the peptides. The most abundant peptide ions are

subjected to MALDI-TOF / TOF analysis, providing information that can be used to determine the sequence. The results from both types of analysis are combined and searched using algorithm e.g. Mascot (<http://www.matrixscience.com>) against protein or translated DNA / EST database. The database is then theoretically digested with trypsin and the experimentally generated mass list is compared to theoretically digested database. The match is scored on number of factors, depending on the search program utilized. This method is recommended for the identification of known purified proteins. However there is limited success in identification of proteins that are not in the database, it is generally not suitable for proteins <15 kDa in size, the matches are based on peptide masses and not sequence information and generally only able to suggest post-translational modifications. Amino acid sequencing conclusively confirms the identity of the protein.

3.2 OBJECTIVE

The objective of this chapter is to characterize lignin-degrading enzymes, especially laccase, from the selected fungal isolate *Cerrena unicolor* MTCC 5159, which produced the maximum laccase titer.

3.3 MATERIALS AND METHODS

3.3.1 ORGANISM AND CULTURE CONDITIONS

Cerrena unicolor MTCC 5159, a basidiomycete was the culture used in this study. Modified Low nitrogen (mod LN) medium ([Appendix 8.1.1](#)) was prepared with half-strength seawater. The culture was raised in the same medium for 6 days, homogenized in Omni Macro-homogenizer (Model No.17505, Marietta, GA, United States) for 5 sec and the mycelial suspension was used as inoculum. Erlenmeyer flasks (250 mL) containing 20 mL mod LN medium were inoculated with 2 mL mycelial inoculum (10 % v/v). Cultures were incubated at 30°C under static conditions, in the dark. On day 6, CuSO₄ at 2 mM final concentration was added to the cultures under aseptic conditions to stimulate laccase production. For large-scale cultivation, 3-L Haffkine flasks with 1 L mod LN medium were inoculated with 100 mL of mycelial suspension and incubated as described above.

3.3.2 PRODUCTION OF ENZYME

On day 12 of cultivation when laccase activity reached its maximum, the cultures were vacuum-filtered to separate the mycelium. The culture filtrate was collected after passing through a series of filter papers namely; Whatman no. 1, GF/C, 0.45 µm and finally 0.22 µm (GV, Millipore, USA). The filtrate was used as the starting material for further experiments. The fungal biomass was estimated as mycelial dry weight ([Appendix 8.2.1.5](#)).

The fungus is known to produce exopolymeric substances (EPS) to up to 50 % of its dry weight ([D'Souza et al., 2006](#)). During protein purification through chromatographic columns, this EPS, if not removed can lead to their blockage. Therefore, it was preferable to remove the EPS to prolong the life of the column. To remove the EPS, the culture filtrate was frozen at -20°C, thawed, and then centrifuged at ~ 22,000 g for 15 min at 4°C. The precipitated EPS was removed.

3.3.3 ENZYME ASSAY

Laccase activity was estimated spectrophotometrically using the ABTS method (Appendix 8.2.2.1.1). It was also estimated by syringaldazine and guaiacol, in specific experiments which warranted it, the estimation procedures are given in Appendix 8.2.2.1.2 & 8.2.2.1.3 respectively. An abiotic control without laccase had been used in every experiment and all the assay components had been brought to reaction temperature before assay. Laccase was expressed as either U L⁻¹ or U mL⁻¹ and has been defined in Appendix 8.2.2.1.1. Protein concentration was estimated spectrophotometrically using Bradfords reagent (Sigma, St. Louis, Montana, U.S.A), (Appendix 8.2.1.4). All spectrophotometric measurements were carried out using UV-Vis 2450 spectrophotometer (Shimadzu, Japan). All values represent the mean of three measurements of two independent experiments each.

3.3.4 COMPARISON OF ENZYME CONCENTRATION TECHNIQUES

Various techniques to concentrate laccase from 250 mL the culture filtrate were compared for obtaining the maximum laccase activity.

3.3.4.1 Concentration with Poly Ethylene Glycol (PEG) 6000: The culture filtrate was placed in pretreated (as per manufacturers protocol) dialysis bags (12 kDa cut off, Sigma). Both the ends were sealed with clips and covered with PEG. The PEG was removed by washing the outer surface of the bag with distilled water. The volume of the retanate was brought up to 10 mL using distilled water.

3.3.4.2 Vacuum Concentration: The culture filtrate was concentrated in a speed vacuum concentrator (Ecospin 314, Biotron, Korea) to 10 mL.

3.3.4.3 (NH₄)₂SO₄ Precipitation: Ammonium sulphate at 80 % saturation (140.25 g) was added slowly to the culture filtrate with stirring at 4°C. After the (NH₄)₂SO₄ had completely dissolved it was stored overnight in the cold, to allow the protein to precipitate. The solution was then centrifuged at ~ 22,000 g at 4°C for 30 min. The

precipitate was rinsed with distilled water and the final volume was brought to 10 mL using distilled water.

3.3.4.4 Ultra Filtration:

3.3.4.4.1 Centrifugal force (Centricon): The culture filtrate was centrifuged in a Centricon with a 5 kDa cutoff membrane at ~ 7,200 g at 4°C for 30 min till 10 mL of retentate was left behind.

3.3.4.4.2 Positive pressure (Amicon): The culture filtrate was concentrated to 10 mL, in an Amicon unit (350 mL capacity, with a 5 kDa cutoff membrane) with constant stirring at 4°C. The membrane cutoff was 5 kDa. Positive pressure was applied using nitrogen gas, to drive the ultrafiltration process.

3.3.5 PURIFICATION OF LACCASE

The Amicon concentrated culture filtrate was considered as the partially purified laccase source. This was filtered through 0.22 µm sterile filter (Whatman Asia Pacific, Singapore) and loaded on a High Load 16 / 60 Superdex 75 preparative grade column and eluted with Na-acetate 0.2 M buffer (pH 4.5) containing 1 M KCl at a flow rate of 1 mL min⁻¹ using a fast protein liquid chromatography system (FPLC; 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 3 laccase peaks obtained during this size exclusion chromatographic step, the fractions showing maximum absorbance at 280 nm and maximum laccase activity, at retention volume of 50 mL (mol mass ~56 kDa) were collected, pooled and concentrated using Centricon with a 5 kDa cutoff membrane (Millipore, MA, USA).

The concentrate was then applied to Mono QTM 10 / 100 GL (Amersham Biosciences, Sweden) 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 peaks showing laccase activity, the laccase fractions eluting at 0.185 - 0.197 M KCl gradient showing

maximum absorbance at 280 nm and maximum laccase activity were pooled and concentrated as above. This laccase fraction was used for further characterization reported in the present study.

3.3.6 CHARACTERIZATION OF LACCASE

3.3.6.1 Non-denaturing SDS PAGE & 2-D PAGE: Non-denaturing SDS PAGE is identical to SDS PAGE (Appendix 8.3.2.1) with the exception that the sample was not heat treated thereby retaining enzyme activity, which could be stained for activity (Appendix 8.3.2.3.1). The molecular mass of the protein could also be determined since SDS was used in the gel and in the sample buffer. For determining the molecular mass, standard protein molecular markers of the medium range 14.3 - 97.4 kDa (Protein molecular mass marker; PMWM, Bangalore Genei, India) were used and protein staining by the silver staining method (Appendix 8.3.2.3.2.1) was performed. The gels were stained for glycoprotein (Appendix 8.3.2.3.3) using the Schiff's reagent (Sigma, MA, USA).

A 2-D PAGE (Bio-Rad, CA, USA) was run for confirming the pI (using IEF strips, pH gradient: 4 - 7) and molecular mass of purified laccase. In case of both non-denaturing PAGE and 2-D PAGE, a 12 % polyacrylamide gel was used.

3.3.6.2 Iso Electric Focussing (IEF): Isozyme pattern of laccase in partially purified mycelia-free culture filtrate was determined by non-denaturing IEF using IEF strips with a pH gradient of 3 - 10 and 4 - 7 in an IEF Cell (BioRad, CA, USA). The strips were stained for activity with guaiacol.

For the purified laccase, denaturing IEF was performed with IEF strips with pH gradient of 4 - 7 and pI was determined by protein (silver) staining.

3.3.6.3 Determination of glycosylation status of purified laccase: For evaluating the *N*-linked glycan content, 0.6 µg purified laccase was treated with 60 mU of Endoglycosidase H (Endo H from *Streptococcus plicatus*, Sigma, MA, USA) overnight at 37°C. A non-denaturing SDS-PAGE of the Endo H-treated and untreated

purified laccase was then carried out. The gel was stained for protein and glycoprotein. The difference in molecular mass of the Endo H-treated and untreated purified laccase, gave the mass (kDa) of the purified laccase that was contributed by its *N*-linked glycan content.

3.3.6.4 Spectral profile of purified laccase: The UV-visible spectrum of purified laccase at 0.7 mg mL⁻¹ was spectrophotometrically determined.

3.3.6.5 Determination of N-terminal amino acid sequence of purified laccase: The purified laccase was electro blotted (by over night transfer at 4°C) from an SDS-PAGE gel onto a PVDF membrane (BioTrace, Pall Pharmalab Filtration, India) using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, CA, USA). The membrane was stained for protein by the Coomassie blue staining method ([Appendix 8.3.2.3.2.2](#)). The band corresponding to the purified laccase was excised and loaded directly onto the automated protein sequencing system (Procise HT - 491, Applied Biosystems, CA, USA), to determine the N-terminal sequence.

3.3.6.6 Determination of internal peptide sequence of purified laccase: Trypsin is a highly active and specific protease, which cleaves the C-terminal to lysine and arginine residues of a protein to generate peptides that fall within the mass range of 500 to 5000 Da, which is suitable for peptide mass mapping. After the *in-gel* digestion of purified laccase, by trypsin, the peptides were extracted by sonication and the spotted on the α -cyano-4-hydroxycinnamic acid (HCCA) matrix for a MALDI TOF analysis. This was carried out at the Proteomics Facility, TCGA, New Delhi with Ultra Flex MALDI TOF, (Germany).

3.3.6.7 Physical properties of laccase:

3.3.6.7.1 Determination of pH and temperature assay optima for laccase: To determine the pH and temperature optima of laccase, its activity was measured with 1mM ABTS prepared in varying pH using different buffer systems. The buffers included glycine-HCl (pH 2.5 and 3), sodium citrate-phosphate (pH 3 - 6),

Tris-HCl (pH 7 - 9) and glycine-NaOH (10 and 11), all of which were prepared at 0.2 M. Laccase activity was assayed at different temperatures for each pH tested. For partially purified laccase assay, the pH and temperature range determined was from 3 - 11 and 5° – 90°C whereas, for purified laccase it was from 2.5 - 9 and 20° – 90°C, respectively using ABTS. For purified laccase, the optimum pH for assay was also determined using different substrates (ABTS, syringaldazine & guaiacol) at 30°C, in the pH range of 2.5 - 9.

3.3.6.7.2 Determination of pH and temperature stability of purified laccase:

To determine the pH stability, purified laccase was preincubated at pH 2.5 – 9 for 1 h at 30°C, the residual activity was estimated with ABTS at pH 3 and 70°C (optimum pH and temperature for activity). For testing temperature stability, it was incubated in Tris-HCl buffer, pH 9 (at its maximum stability) at 50°, 60° and 70°C for various incubation periods. Activity was then estimated as above. It was presented as % relative activity, considering activity at 0 h to be 100 %.

3.3.6.8 Kinetic properties of purified laccase:

3.3.6.8.1 Determination of temperature coefficient (Q_{10}) & Energy of Activation (E_{act}) values:

The Q_{10} & E_{act} values of purified laccase were determined based on the pH & temperature assay results. The formulae used for calculations are given in [Appendix 8.2.3.2.5](#) & [8.2.3.2.6](#) respectively.

3.3.6.8.2 Determination of maximum velocity (V_{max}), Michaelis constant (K_m), catalytic constant (K_{cat}) & specificity constant:

The V_{max} and K_m were determined from the Lineweaver Burke plot pH 3 at 30° & 70°C for the substrate ABTS and at pH 6 (optimum pH) and 30°C for the substrate syringaldazine. The enzyme assay was carried out at varying substrate concentrations; 10 - 500 μ M for ABTS and at 10 - 200 μ M for syringaldazine. The K_{cat} and specificity constant were determined from the K_m and V_{max} values. The calculations for V_{max} , K_m , K_{cat} and specificity constant are given in [Appendix 8.2.3.2.1 - 8.2.3.2.4](#).

3.3.6.9 Catalytic properties of purified laccase:

3.3.6.9.1 Substrate specificity and inhibitors: Oxygen uptake during the reaction of purified laccase with various substrates, non-substrates, mediators and inhibitors was measured for 15 min using an oxygen electrode (Oxygraph, Hansatech, Norfolk, England) at 30°C in a 1 mL reaction chamber. The total volume was made to 1 mL with 0.2 M citrate phosphate buffer (pH 4), such that there was no air gap between the under surface of the lid and the surface of the reaction mixture. The reaction mixture for the testing of substrates consisted of 1 U purified laccase and the substrate. Whereas for the testing of non-substrates, mediators and inhibitors, the reaction mixture contained 1 U purified laccase, 1 mM ABTS and the compound under test. In case of inhibitors, the purified laccase was pre-incubated with the inhibitor for 1 min before the addition of 1 mM ABTS, after which oxygen consumption was monitored. The rate of oxygen uptake was measured in terms of $\text{nmol O}_2 \text{ min}^{-1} \text{ U}^{-1}$ laccase. The activity was expressed in terms of % relative activity, considering laccase activity in the presence of only 1 mM ABTS as 100 %. The composition of the control for both substrate specificity and inhibitor effect was identical to that of the test sample and was subjected to identical conditions, however here purified laccase was absent.

3.3.6.9.2 Effect of metal ions: Effect of various metal ions at 1 mmol on the activity of purified laccase was measured spectrophotometrically using 1 mM ABTS as substrate, at its optimum pH (3) and temperature (70°C). The purified laccase was pre-incubated for 1 min with the metal ion before assaying with ABTS. The activity was expressed as % relative activity considering the activity of purified laccase in the presence of only ABTS as 100 %. The control contained the metal ion dissolved in glycine-HCl buffer (pH 3) in the same volume used to dissolve laccase and was pre-incubated at 70°C for 1 min and was assayed without the presence of purified laccase.

3.4 RESULTS

Cerrena unicolor MTCC 5159 showed growth and laccase production in mod LN medium prepared with sea water (Fig. 2). Laccase activity and fungal biomass (growth) reached a maximum on day 12 (~12,600 U L⁻¹) hence, the culture was regularly harvested for laccase on day 12 of growth, for purification and characterization purposes.

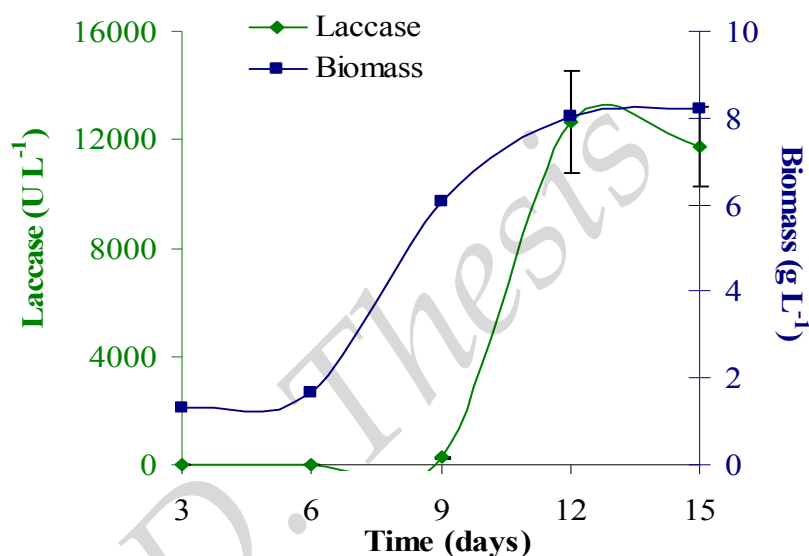


Fig. 2: Temporal production of laccase and biomass.

Table 1: Efficiency of different concentration techniques.

Concentration Technique	Laccase Activity (U mL ⁻¹)	Protein Concentration (mg mL ⁻¹)	Specific Activity (U mg ⁻¹)	Fold
<i>Culture Filtrate</i>	24	0.1	330	1
<i>PEG Concentration</i>	775	1.5	2260	33
<i>(NH₄)₂SO₄ Precipitation</i>	515	2	1445	22
<i>Ultrafiltration (centrifugal)</i>	430	1.5	1700	18
<i>Ultrafiltration (positive pressure)</i>	3245	0.3	12981	137
<i>Vacuum Concentration</i>	735	2.5	1590	31

Various methods to concentrate the culture filtrate were compared. These included concentration by polyethylene glycol (PEG) 6000, ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$, ultrafiltration by positive pressure (Amicon) and by centrifugal force (Centricon) and by vacuum concentration (Table 1). The culture filtrate was concentrated exactly 25 times for all the concentration techniques used. It is clear that Amicon concentration was the most efficient method for the concentration of laccase, it also yielded maximum specific activity. Henceforth, Amicon concentrated culture filtrate was used as the source of partially purified laccase for all other experiments.

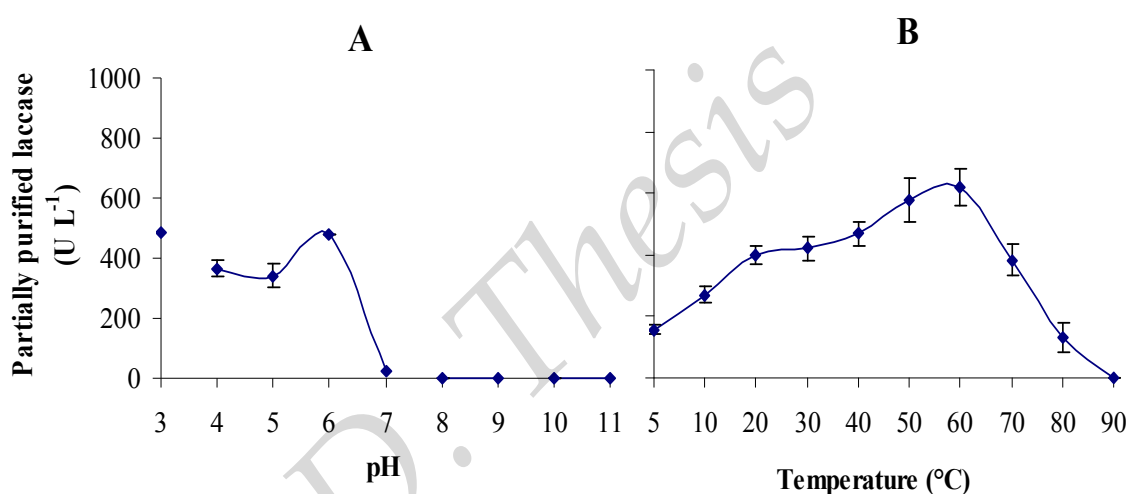


Fig. 3: Effect of (A) pH and (B) Temperature on assay of partially purified laccase.

The pH and temperature optima (Fig. 3A & B) for assay of partially purified laccase were determined spectrophotometrically, using ABTS as substrate. Laccase showed a bimodal pH profile with activity peaks at both 3 and 6. However with slightly greater activity at pH 3 (Fig. 3A) and maximum activity at 60°C (Fig. 3B). Partially purified laccase preparations were estimated at pH 3 and 60°C, henceforth.

Non-denaturing IEF was first carried out in the pH range of 3 - 10 (data not shown) the laccase bands obtained were within the pH range of 4 – 7. Due to the broad pH range, the bands obtained were close to each other and not very distinguishable. Hence a non- denaturing IEF in the pH range of 4 – 7 (Fig. 4) was carried out and the strip stained for activity with guaiacol. Two very distinct laccase bands were observed

besides several other laccase bands, which were not clearly distinguishable. This suggested the presence of several laccase pI isozymes (seen as fine bands due to their relative lower concentration) to the tune of over 20, in partially purified laccase.



Fig. 4: Activity staining by guaiacol after non-denaturing IEF (pH 4 - 7) of partially purified laccase.

Due to the presence of a large number of laccase isozymes based on pI, a size exclusion chromatographic step was carried out prior to anion exchange chromatography. When partially purified laccase was subjected to size exclusion chromatography (Superdex-75 column), it yielded 3 distinct laccase peaks corresponding to molecular mass of 82, 56 and 45 kDa termed as Lac I, Lac II and Lac III (Fig. 5) respectively. Compared to the other isozymes, Lac II was present in maximum abundance.

Hence it was selected for further resolution by anion exchange chromatography (Mono Q column). This resolved into 4 major peaks (Fig. 6), indicating that at least 4 different isozymes having the same molecular mass but differing in surface charge were present. Lac I & III were also resolved into 4 peaks each, by anion exchange chromatography (data not shown). This indicates that all the three laccases have isozymes differing in their surface charges. Due to significantly low recovery, these laccases were not characterized.

From the anion exchange chromatography of Lac II, the peak showing maximum absorbance at 280 nm also corresponded to maximum laccase activity (eluted between 0.185 and 0.197 M KCl gradient) was designated '**Lac IId**'. This laccase isozyme was selected for further characterization.

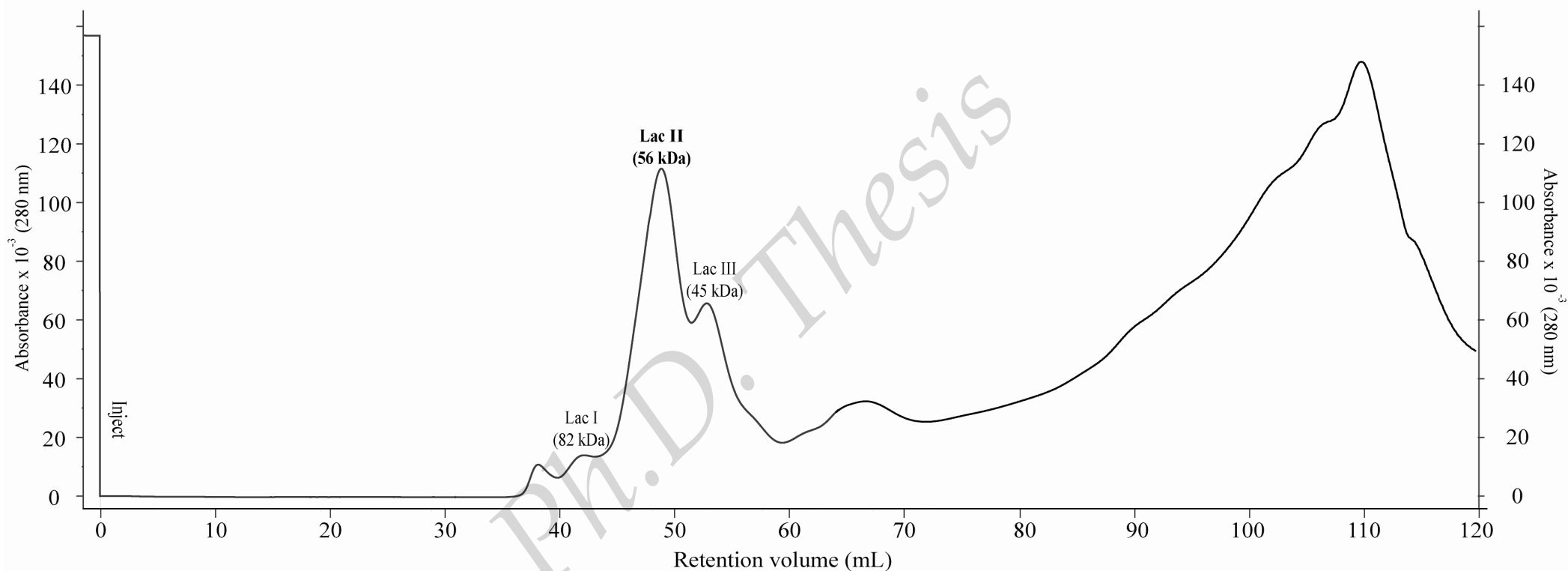


Fig. 5: Size exclusion chromatographic profile of crude laccase using Superdex 75 column.

Culture filtrate of a 12 day-old culture of *Cerrena unicolor* MTCC 5159 grown in mod LN medium was concentrated by ultrafiltration (positive pressure) and used as the source of crude laccase. 3 major peaks Lac I, Lac II and Lac III with differing molecular masses were obtained.

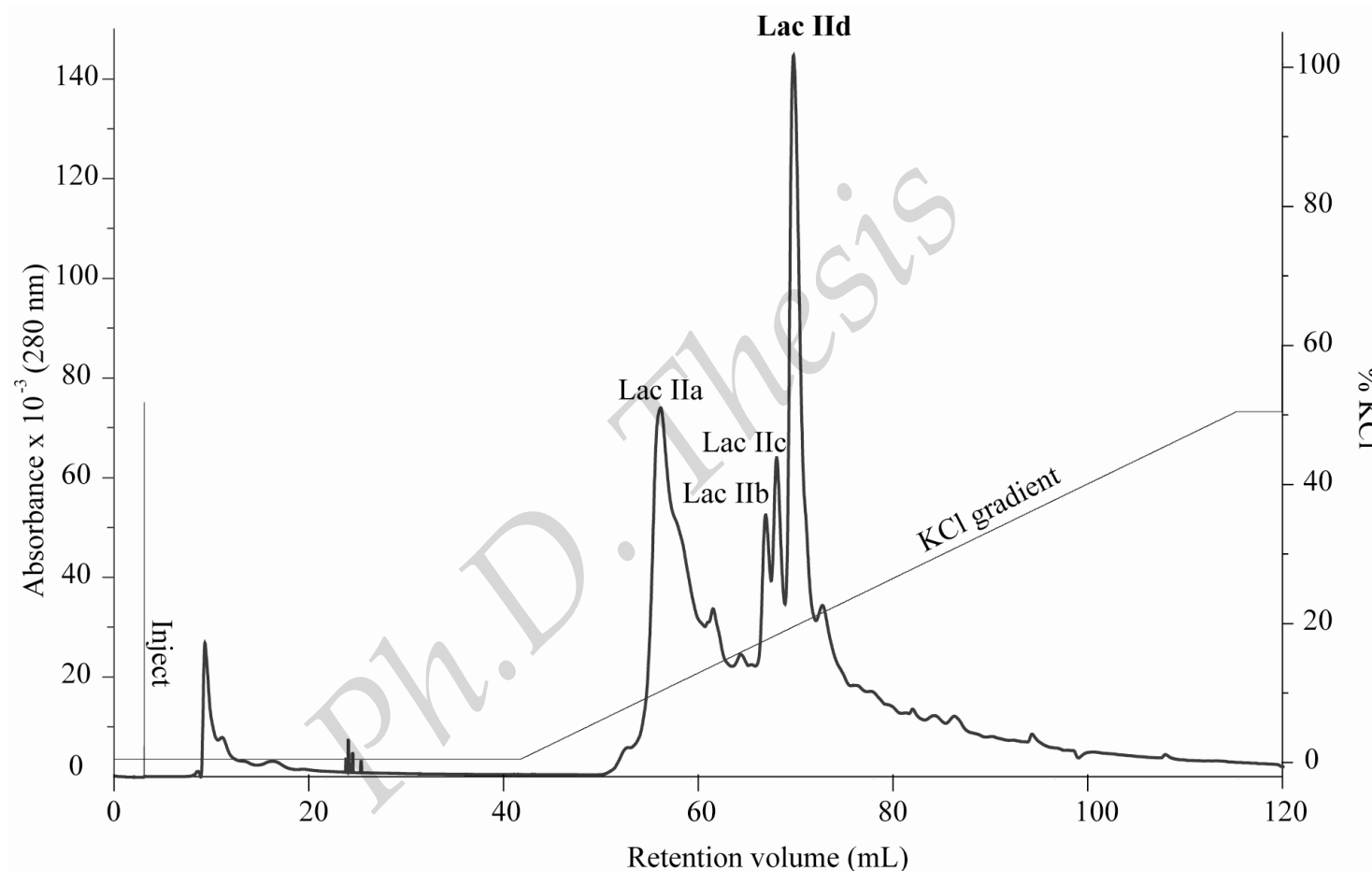


Fig. 6: Anion exchange chromatographic profile of Lac II fraction from the size exclusion chromatographic step, using Mono Q column. The KCl gradient was maintained between 0 to 0.5 M. Lac II resolved into 4 peaks with differing surface charges namely Lac II_a, II_b, II_c and II_d.

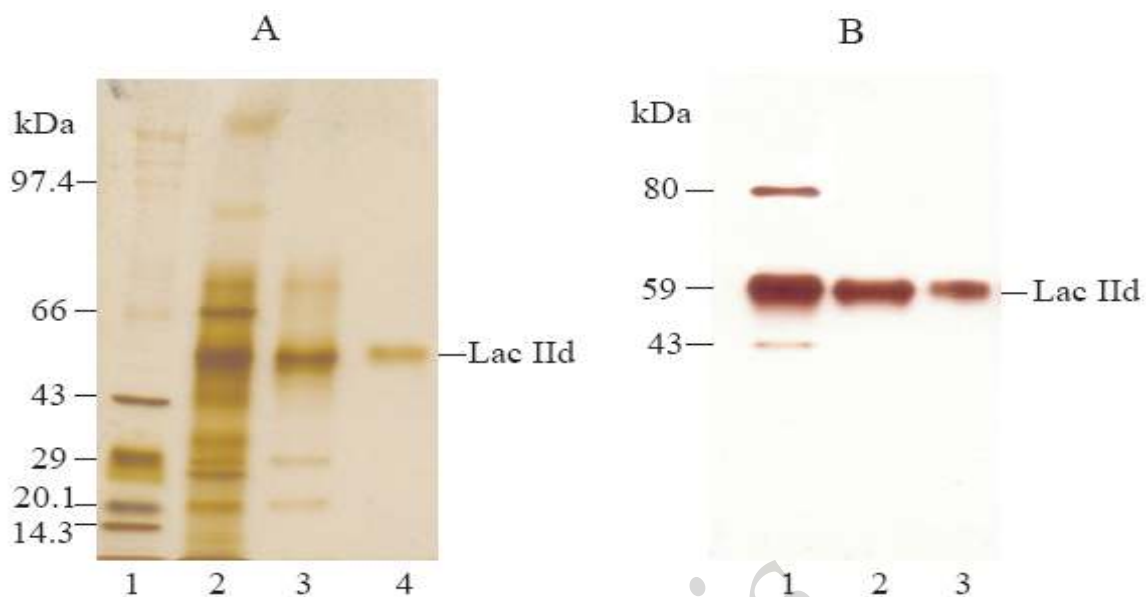


Fig. 7: Non-denaturing SDS-PAGE of *Cerrena unicolor* MTCC 5159 laccase. (A) Silver and (B) Guaiacol staining of the 12 % gel.

Lanes 1, 2 & 3 of (B) correspond with lanes 2, 3 & 4 of (A), respectively

Lane 1: Protein molecular mass marker; lane 2: concentrated partially purified laccase; lane 3: Lac II (from size exclusion chromatography); lane 4: Lac II d (from anion exchange chromatography).

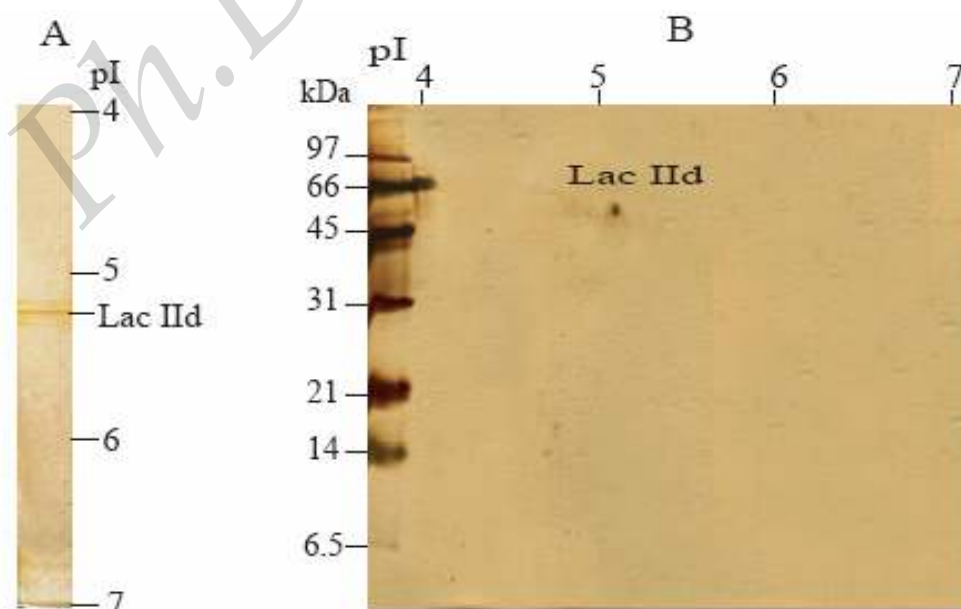


Fig. 8: Confirmation of Lac II d homogeneity. Silver staining of (A) IEF strip, pH 4 - 7 and (B) Gel from 2D PAGE.

A non-denaturing SDS PAGE for the laccase samples obtained at each purification stage was performed. The gel was stained for total protein (Fig. 7A) and activity (Fig. 7B). Activity staining of the gel confirmed that at least 3 laccase molecular mass isozymes were present in partially purified laccase with masses of 80, 59 and 43 kDa which corresponded to the 82, 56 and 45 kDa laccase peaks obtained during size exclusion chromatography. The Lac II fraction further resolved into several laccase isozymes including Lac IId, which had a mass of 59 kDa from Fig. 7A.

The pI was determined to be 5.3 by IEF (Fig. 8A). Besides confirming the molecular mass and pI of Lac IId, 2D PAGE (Fig. 8B) which had been carried out under denaturing conditions (involving heat) revealed that Lac IId was monomeric in nature.

Table 2: Purification of Lac IId from the culture filtrate of *C. unicolor* MTCC 5159.

Purification Step	Total Laccase (U)	Total Protein (mg)	Specific Activity (U mg⁻¹ protein)	Yield (%)	Purification Factor (fold)
<i>Culture Filtrate</i>	23714	72	330	100	1
<i>UltraFiltration</i> (5 kDa cut off)	129810	12	11126	547	34
<i>Size Exclusion Chromatography</i> (Superdex 75)	3039	0.92	3315	2	10
<i>Anion Exchange Chromatography</i> (Mono-Q)	531	0.05	10918	17	33

A summary of the purification level obtained at each step is given in Table 2. A 33 - fold purification with a final yield of 17 % for Lac IId was achieved

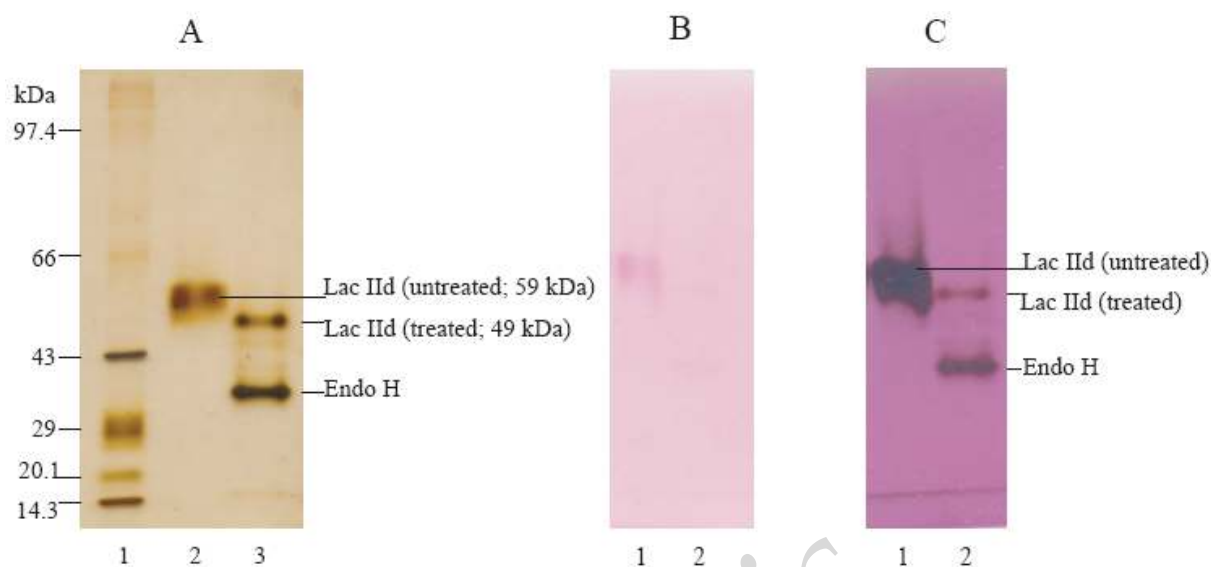


Fig. 9: Glycosylation status of Lac IId; Non-denaturing SDS-PAGE (12 %) for Endo H-treated Lac IId; (A) Silver staining; (B) Glycoprotein (Schiff's) staining and (C) Silver staining of the glycoprotein-stained gel.

Lane 1: Protein molecular mass marker; lane 2: Lac IId (untreated); lane 3: Lac IId treated with Endo H. Lane 1 & 2 of (B) and (C) correspond to lane 2 & 3 of (A) respectively.

In order to determine the percentage of molecular mass contributed by *N*-linked glycosylation, Lac IId was treated with Endoglycosidase H (Endo H) that selectively removes *N*-linked glycosylation. The molecular mass of Lac IId was reduced to 49 kDa (Fig. 9A) revealing that Lac IId contained an *N*-linked glycan content of 17 %. The untreated Lac IId showed positive reaction for glycosylation with Schiff's reagent (Fig. 9B), whereas, the Endo H-treated sample tested negative, thus Endo H was successful in removing most of the glycosylation of Lac IId, as seen in Fig. 9B. Almost the entire glycosylation was of the *N*-linked type. Silver staining of the Endo H-treated Lac IId, after it had been stained for glycosylation was carried out to confirm the presence of Endo H-treated Lac IId in the gel (Fig. 9C).

The UV-visible spectrum of Lac IId (0.7 mg mL^{-1}) demonstrated a peak at 610 nm and a shoulder at 330 nm (Fig. 10) which is typical of blue laccases.

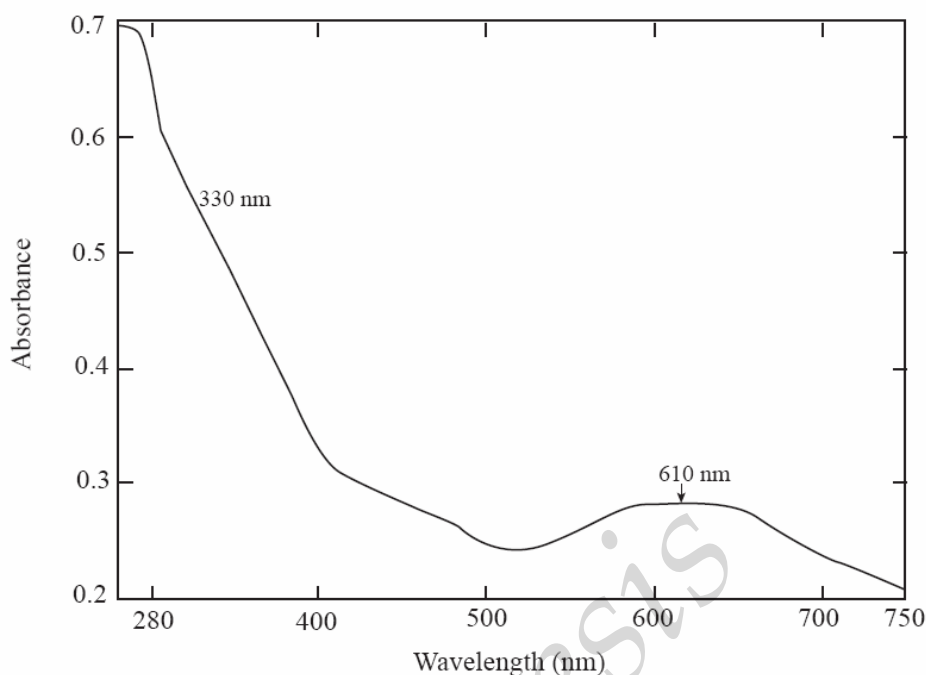


Fig. 10: UV-visible spectrum of Lac IId.

Table 3: Alignment of the Lac IId N-terminal sequence with other fungal laccases.

	1	10	20	30	Similarity (%)	E value	
<i>Cerrena unicolor</i> MTCC 5159 (P85430)	GT	GPVADLHI	INKDLS	PDGFQ	PTVVAGGG	100	6e-19
<i>Schizophyllum commune</i> (BAA31217)	AL	GPVGNLPI	VNKEI	IAPDGF	SPTVLANGK	85	8e-06
<i>Spongipellis</i> FERMP 18171 (BAE96003)	AI	GPVADLHI	VDVSI	IAPDGF	SPPAVLAGGT	85	5e-06
<i>Panus rudis</i> (AAW28932)	AI	GPVTDLHI	VNDNI	IAPDGF	TFAAVLAGGT	81	8e-06
<i>Rigidoporus microporus</i> (AAO38869)	AI	GPVADLHI	SNANIS	PDGF	TFAAVLAGGS	81	6e-06
<i>Volvariella volvacea</i> (AAR03582)	AI	GPVTELQ	IVNDEI	IAPDGF	SFGSVLANGA	77	0.018
<i>Trametes</i> I-62 (AAQ12270)	AV	GPVTDLT	ISNANV	SPDGF	QFAAVVANGG	75	3e-05
<i>Ganoderma lucidum</i> (AAR82930)	AI	GPVANLT	ISDADI	IAPDGF	TFAAVVNVG	70	0.25
<i>Cerrena maxima</i> (2H5U)	GV	GPVADNT	ITNAATS	PDGF	SQAVVNVG	58	1.7
	.	*** :	* :	:**** *	:*:.*		

The N-terminal amino acid sequence of Lac IId was deposited in the universal protein resource database, 'Uniprot' (<http://www.pir.uniprot.org>) under the accession number P85430. A comparative study of homology of various laccase N-terminal sequences with P85430 using the BLASTP algorithm, at the website: <http://blast.ncbi.nlm.nih.gov/Blast>, was conducted. This was then aligned using the Clustal version T-Coffee v. 5 (www.tcoffee.org). Similarity values were determined by algorithm 'bl2seq' (Tatusova &

Madden, 1999) where each of the N-terminal sequences given in Table 3 were aligned with P85430.

Comparison with other laccases showed 70 - 85 % similarity with basidiomycetous fungi (Table 3). Maximum similarity was observed with laccases from *Schizophyllum commune* and *Spongipellis* sp. that are white-rot basidiomycetous fungi.

From the MALDI TOF analysis of Lac IId, the sequence of only one peptide could be positively identified. This internal peptide sequence was deposited in 'Uniprot' under the accession number P85430 (same as the N-terminal sequence). A comparative study of various internal peptide sequences homologous to P85430 internal peptide using the HSSP (Homology Derived Secondary Structure of Proteins, v. 1.1, 2001) was conducted, after digesting the databases (protein, translated DNA & EST) with trypsin *in-silico*. P85430 matched with several known laccases. The maximum score of 51 % at $p = 0.05$; using the algorithm, 'MASCOT Search' of Matrix Science (available at <http://www.matrixscience.com/>) was with a peptide (Q69FX1_9AGAR) present in 'Laccase 2' of *Volvariella volvacea* (AAR03581, from NCBI database). The scoring is based on the 'Probability based Mowse Score' which is the 'Ion score' being equal to $10 \times \log(P)$, where P is the probability that the observed match is a random event. Individual ions scores > 42 indicate identity or extensive homology at $p < 0.05$. Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. The internal peptide, P85430 of *Cerrena unicolor* MTCC 5159 had 100 % homology with Q69FX1_9AGAR peptide of Laccase 2 of *Volvariella volvacea* (AAR03581) at position 471. However, AAR03581 has 545 amino acids (AAs) and P85430 has only 17 AAs, resulting in sequence coverage of only 2.75 %, which is not enough. The predicted molecular mass and pI for AAR03581 was 59367 Da (~59 kDa) and 5.4, respectively, similar to Lac IId of *C. unicolor* MTCC 5159 values for molecular mass and pI.

These sequences were aligned using the Clustal version T-Coffee v. 5 and presented in Table 4. Varying colours denote varying kinds of amino acids (AAs), those which are presented in an identical colour, have similar functions. Only the sequences showing maximum similarity to P85430 were considered, three exceptions were made; *Trametes* sp. I-62 (AAQ12270) which was chosen since it was also used in Table 3, *Ganoderma lucidum* (AAG17009) was chosen on the same basis although the accession

number differed with that of the one used in Table 3. *Cerrena maxima* (2H5U) was chosen for N-terminal as well internal peptide homology studies, since it belonged to the same genus as that of MTCC 5159.

Table 4: Alignment of the Lac IId internal peptide sequence with other fungal laccases.

	1	5	10	15	Signal peptide (AA)	Start Position (AA) including Signal peptide	Similarity (%)	E value
<i>Cerrena unicolor</i> MTCC 5159 (P85430)							100	
<i>Volvariella volvacea</i> (AAR03581)	RDVVS	IGR	--	AGDNVTIRF	1-22	471	100	3e-07
<i>Rigidoporus microporus</i> (AAO38869)	RDVVS	IGN	--	AGDNVTIRF	1-21	444	94	2e-05
<i>Flammulina velutipes</i> (BAE91880)	RDVVS	IGA	--	AGDNVTIRF	--	423	94	2e-05
<i>Laccaria bicolor</i> (XP_001886681)	RDVVS	IGG	--	AGDNVTIRF	1-17	439	94	4e-05
<i>Pholiota nameko</i> (ABR24264)	RDVVS	IGG	--	AGDNVTIRF	1-18	444	94	4e-05
<i>Panus rudis</i> (AAW28932)	RDVVSTGT	--	AGDNVTIRF		1-21	445	88	3e-04
<i>Trametes</i> sp I-62 (AAQ12270)	RDVVSTGT	PA	AGDNVTIRF		1-25	448	--	0.008
<i>Cerrena maxima</i> (2H5U)	RDVVSTGT	PA	AGDNVTIRF		--	423	--	0.008
<i>Ganoderma lucidum</i> (AAG17009)	RDVVSTGT	PA	AGDNVTIRF		1-21	444	--	0.008
	*****	*		*****				

Comparison of the internal peptide, P85430 with the correspondingly-positioned internal peptides of other basidiomycete laccases showed 88 - 100 % similarity (Table 4). Similarity values were determined in the same way as for the N-terminal sequence. 100 % similarity was observed with the Q69FX1_9AGAR (*in-silico* tryptic digest), an internal peptide of ‘Laccase 2’ from *Volvariella volvacea* (AAR03581). *Panus* had a homology of 88 % with the internal peptide, P85430 (Table 4) and 81 % with the N-terminal, P85430 (Table 3). *Trametes*, *Ganoderma* and *Cerrena* did not show significant homology with the internal peptide P85430, enough to be listed, whereas they showed the lowest homology of 75, 70 and 58 %, respectively with the N-terminal, P85430 (Table 3).

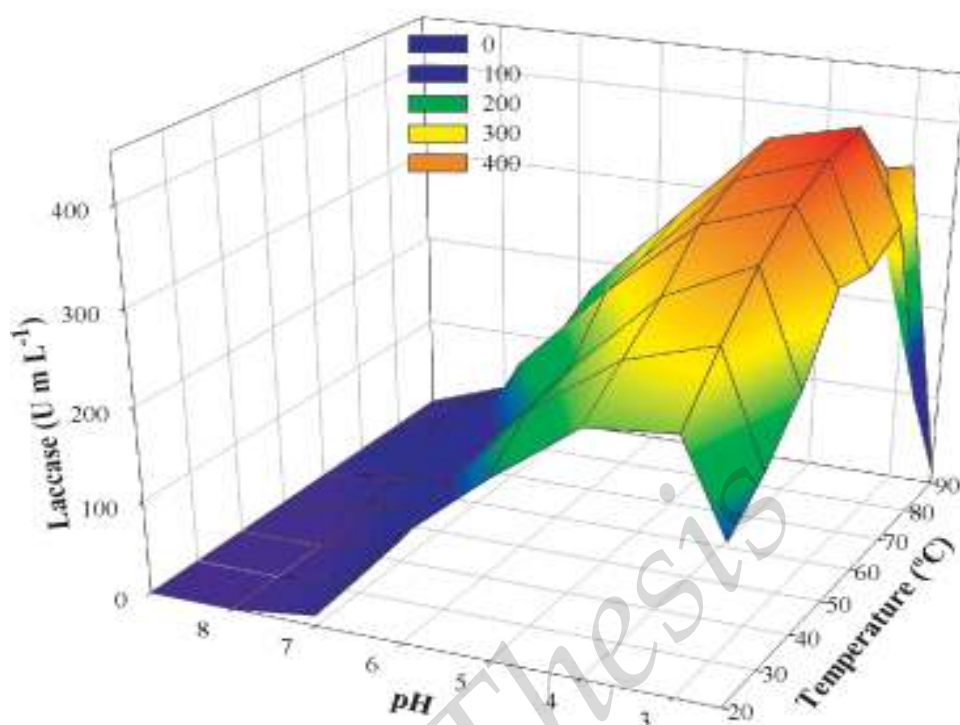


Fig. 11: Effect of pH and temperature on assay of Lac IId.

Lac IId (purified laccase isozyme) showed optimum activity at pH 3 and 70°C (Fig. 11). Whereas with guaiacol and syringaldazine, Lac IId had optimum activity at pH 6 (Fig. 12A).

The enzyme retained 100 % of its activity after 1 h at pH 9 and 30°C and thus was most stable at this pH (Fig. 12B). When Lac IId was incubated at pH 9 in Tris-HCl buffer for 1 h at various temperatures, it retained about 60 % of its activity at 70°C (Fig. 12C). At this pH, more than 60 % of activity was retained at 50° and 60°C for up to 180 min whereas about 50 % residual activity was detected up to 90 min at 70°C (Fig. 12D).

The shelf life of Lac IId is presented in Fig. 12E. Lac IId retained 67 % and 100 % of its activity at 4° and -20°C respectively for over 35 days and complete activity even after 1 year of storage at -20°C.

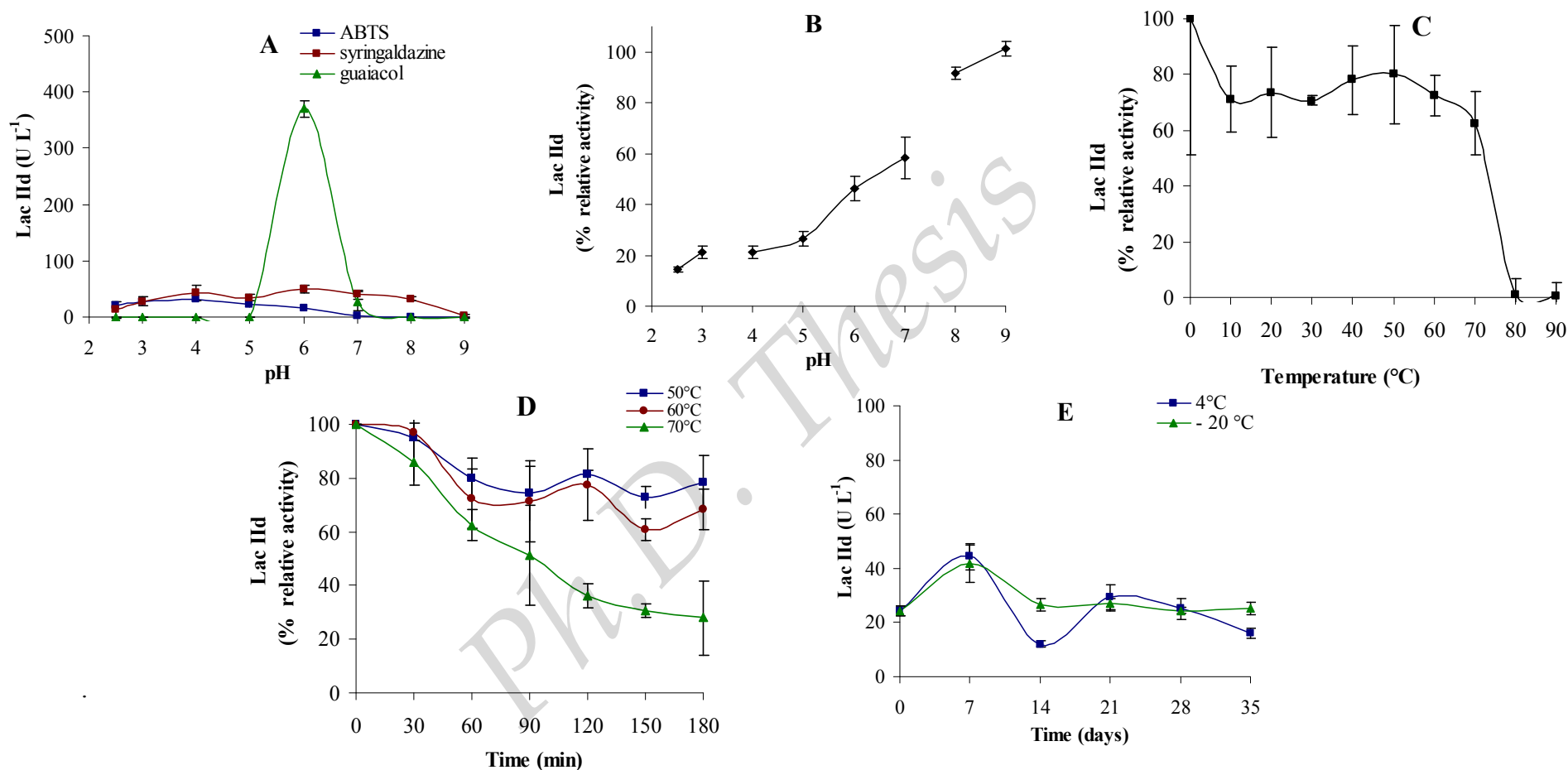


Fig. 12: Properties of Lac II d. (A) Effect of pH on assay (using various substrates at 30°C); (B) pH stability (after incubation at 30°C for 1 h at varying pH); (C) Thermostability between 0° – 90°C (after incubation at pH 9 for 1 h); (D) Thermostability between 50 °- 70°C (after incubation for various time intervals at pH 9); (E) Shelf life at 4° and -20°C (when stored at pH 9).

Table 5: Q₁₀ values for Lac IId at temperatures (20° - 90°C) and pH (2.5 - 8).

pH	2.5	3	4	5	6	7	8
Q ₂₀₋₃₀	1.21	1.20	1.14	1.20	1.13	1.42	ND [#]
Q ₃₀₋₄₀	1.24	1.15	1.12	1.05	1.10	1.35	ND [#]
Q ₄₀₋₅₀	1.09	1.09	1.14	1.22	1.43	1.52	ND [#]
Q ₅₀₋₆₀	0.97	1.05	1.06	1.03	1.00	1.03	2.14
Q ₆₀₋₇₀	1.07	1.02	1.05	1.07	1.14	1.31	3.20
Q ₇₀₋₈₀	0.39	0.85	0.75	0.87	0.77	0.81	0.94
Q ₈₀₋₉₀	0.11	0.95	1.05	0.72	0.83	0.66	0.71

[#] Where the activity was zero, it was not possible to calculate the Q₁₀ value (hence ND, not determined). Since showed no activity was observed at pH 9, at any temperature it was not included in the table.

The Q₁₀ values were found to be >1 between 20° and 70°C in the pH range of 3 - 7 for Lac IId (Table 5). The E_{act} for Lac IId was found to be 13.4 k J mol⁻¹ at pH 3 between temperature 20° and 30°C which decreased to 2.5 kJ mol⁻¹ between 60° and 70°C, at its optimum pH (3).

Table 6: Kinetic parameters of Lac IId. *

Substrate	K _m (μM)	V _{max} (μmol min ⁻¹)	K _{cat} (min ⁻¹)	Specificity Constant (K _{cat} / K _m) (min ⁻¹ μM ⁻¹)
<i>ABTS</i> [#] (70°C)	54	2	6507	120
<i>ABTS</i> [#] (30°C)	57	2	5327	93
<i>Syringaldazine</i> [#] (30°C)	19	1	2112	110

*The amount of Lac IId was held constant (0.0196 μg) in all the assays.

[#]Assay carried out at optimum pH.

The K_m, V_{max} and K_{cat} of Lac IId were compared using the substrates 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 6).

Table 7: Oxidation of various substrates (1 mM) by Lac IId.

Substrates	% Relative Activity (SD)
ABTS	100 (27)
Ferulic acid	64 (9)
Guaiacol	53 (7)
Syringaldazine	42 (9)
L-ascorbic acid	33 (13)
N,N-dimethyl- <i>p</i> -phenylenediamine	21 (2)
Pyrocatechol	15 (3)
Indulin (0.25 %)	15 (7)
<i>p</i> -anisidine	0 (0)
2,5-dimethyl aniline	0 (0)
Violuric acid	0 (0)
*L (-) tyrosine	0 (0)
Vanillic acid	0 (0)

*L (-) Tyrosine is a substrate of tyrosinase and not laccase.

SD = Standard deviation

Among the substrates (Table 7), ABTS was found to be the most preferred substrate followed by ferulic acid and guaiacol. Tyrosine, vanillic acid, 2,5-dimethyl aniline, *p*-anisidine and violuric acid were not oxidized by Lac IId.

Table 8: Oxidation of various non-substrates (1 mM) by Lac IId, in presence of ABTS (1 mM) as mediator.

Non Substrates	% Relative Activity (SD)
<u>Control (only ABTS)</u>	100 (11)
<i>p</i> -anisidine	161 (12)
2,5-dimethyl aniline	132 (16)
Violuric acid	122 (16)
L (-) tyrosine	82 (7)
Vanillic acid	67 (8)

SD = Standard deviation

However in the presence of ABTS with the exception of vanillic acid and tyrosine, most of the compounds which previously did not get oxidized (Table 8) got oxidized.

Table 9: Oxidation of various known mediators (1 mM) by Lac IId, in presence of ABTS (1 mM) as substrate.

Mediators	% Relative Activity (SD)
<u>Control (only ABTS)</u>	100 (11)
Hydroquinone	160 (21)
3,5-dimethoxy-4-hydroxyacetophenone	128 (23)
Syringic acid	122 (10)
Veratryl alcohol (3,4-dimethoxy benzyl alcohol)	109 (13)
4-hydroxy-2,2,6,6-tetra-methylpiperidin-1-oxyl	100 (15)
1-hydroxybenzotriazole	86 (11)

SD = Standard deviation

When ABTS (1 mM) was used as substrate, four of the known mediators; hydroquinone, 3,5-dimethoxy-4-hydroxyacetophenone, syringic acid and veratryl alcohol (Table 9) were effective in increasing the activity of laccase whereas 4-hydroxy-2,2,6,6-tetra-methylpiperidin-1-oxyl and 1-hydroxybenzotriazole were not effective.

Table 10: Effect of inhibitors on Lac IId, in presence of ABTS (1 mM) as substrate.

Inhibitors	% Relative Activity (SD)
<u>Control (only ABTS)</u>	100 (11)
DTT	160 (7)
#4-hexyl resorcinol	117 (10)
Cysteine	115 (9)
EDTA	104 (4)
Cysteine (0.1 mM)	104 (7)
<i>o</i> -coumaric acid	101 (5)
DTT (10 mM)	80 (3)
8-hydroxyquinoline	77 (10)
4-nitro phenol	73 (7)
CTAB	69 (5)
Kojic acid	62 (3)
Tropolone	58 (9)
Sodium chloride (1M)	30 (4)
Potassium chloride (1M)	30 (6)
2-mercapto ethanol	23 (5)
SDS (1%)	23 (0)
Sodium azide (0.1 mM)	5 (3)

#4-hexyl resorcinol is an inhibitor of tyrosinase and not laccase.

Among the various inhibitors tested for Lac IId, sodium azide followed by SDS and mercaptoethanol inhibited its activity (Table 10). It was not inhibited by 4-hexyl resorcinol, a differential inhibitor of tyrosinase but not laccase. The enzyme was neither inhibited by L-cysteine nor DTT, which are known inhibitors of laccase.

Table 11: Effect of metal ions (1 mmol) on Lac IId, in presence of ABTS (1 mM) as substrate.

Metal ions	% Relative Activity (SD)
Control (only ABTS)	100 (11)
#NH ₄ ⁺	104 (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)
Hg ⁺²	68 (2)
Ag ⁺²	66 (15)
Sn ⁺²	63 (5)
W ⁺⁶	52 (1)
Cr ⁺⁴	44 (11)

#Is not a metal ion.

Activity of Lac IId was maximally inhibited by Cr and W by 56 and 48 % respectively, whereas in the presence of Sn, Ag and Hg the inhibition was only ~32 – 37 % (Table 11). The other metal ions did not show significant inhibition.

3.5 DISCUSSION

When comparing various enzyme concentration methods, it was realized that in vacuum concentration inhibiting substances present in seawater (Luo *et al.*, 2005) do not get removed but rather get concentrated. This combined with its low capacity and the long time taken to concentrate, deters one from using this as a method for concentration of laccase on a large scale. Concentration with PEG requires the use of dialysis bags, this method besides being expensive and having a low capacity also requires the pretreatment of the dialysis tubing. The process is also arduous and time consuming. Ammonium sulphate precipitation method also suffers from similar drawbacks, especially the post processing where large volumes of liquid require to be centrifuged and the salt also requires to be completely removed for further processing. Ultrafiltration by centrifugal pressure (Centricon) is relatively easy to use but its low capacity, expensive centrifugal tubes and the rapid blockage of its membranes which cannot be regenerated, hinders its use on a large scale. This however is ideal for laccase concentration during the chromatographic steps where very small volumes of relatively pure laccase fractions are generated and have too low a volume to be used by ultrafiltration with positive pressure (Amicon) concentration method.

It is clear that Amicon concentration was the most efficient among all the methods tested for the concentration of laccase, it also yielded maximum specific activity i.e. several small molecular mass extraneous proteins and inhibiting substances present in the medium were removed. For both Centricon and Amicon, the material used was regenerated cellulose with identical cutoff values yet Amicon was more efficient in concentrating laccase and the membrane although expensive, could also be regenerated several times over. The large capacity of the Amicon unit (can concentrate up to 350 mL at a time) and non-requirement of expensive chemicals, makes it the method of choice to concentrate laccase from the culture filtrate and was selected for all further work. The Amicon concentrated culture filtrate was the source of partially purified laccase for all further experiments.

When partially purified laccase was subjected to non- denaturing IEF (pH range of 4 – 7) and stained for activity, two very distinct and several indistinct laccase bands were observed. This suggested that several laccase pI isozymes (seen as fine bands

due to their relative lower concentration) to the tune of over 20, were present in the concentrated culture filtrate. Since it was a non-denaturing IEF, urea was not used; this to a certain extent affects the determination of the exact pI, since electro-endosmosis could not be avoided, nor the resolution (<http://www.weihenstephan.de/blm/deg/manual/manfrm.htm>). However, the non-denaturing IEF served its purpose in detecting the presence of several laccase isozymes.

Non-denaturing SDS PAGE confirmed that more than one molecular mass laccase isozymes were present. Since there were many more isozymes based on surface charge than molecular mass, a size exclusion followed by an anion exchange chromatographic step was carried out in an attempt to purify laccase.

The size exclusion chromatography corroborated the non-denaturing SDS PAGE results demonstrating the presence of more than one molecular mass laccase isozyme. At least four different isozymes based on surface charge were obtained for each molecular mass isozyme. These results are in concurrence with the non-denaturing IEF (Fig. 4), which shows numerous laccase isozymes based on pI.

Lac IId has 17 % *N*-linked glycosylation, the average range for glycosylation of laccase is between 10 % and 25 % (Baldrian, 2006). *N*-linked glycosylation, plays a structural role in laccase and to a certain extent protects it from extracellular proteases (Yoshitake *et al.*, 1993). This is essential if Lac IId or even the crude or partially purified laccase preparation is to be used in the wild / in-situ, for bioremediation purposes where provision for protection from proteases would be prohibitively expensive.

When *Cerrena unicolor* MTCC 5159 was grown in mod LN medium prepared in seawater, in presence of CuSO₄ the pathway was routed to laccase production with negligible quantities of the peroxidases, which are normally produced albeit in low quantities. This fungus was demonstrated to grow and produce 85,829 U L⁻¹ laccase in high nitrogen medium containing peptone and yeast extract prepared with sea water in the presence of 1 % textile mill effluent (D'Souza *et al.*, 2006). However, a mineral medium like modified LN medium was used since it supported laccase production yet did not increase the load on the purification column. Modified LN medium was ideal for purification and characterization of laccase even though the overall laccase production was lower. It produced a moderately high laccase titer of ~23,700 U L⁻¹ with a specific

activity of 330 U mg⁻¹ protein. This is significant since laccase activity is normally inhibited in the presence of seawater (Luo *et al.*, 2005). This would signify that either the laccases produced are tolerant to the consortium of salts present in sea water or that the actual laccase titer was much higher than that which was estimated by the laccase assay, i.e. the laccase titer would have been actually underestimated. This may provide an explanation for the total laccase, (Table 1) in the culture filtrate was lower than that after ultrafiltration. The inhibiting salts present in the culture filtrate, due to its small size, get removed during the course of ultrafiltration. Thus in the absence of the inhibiting salts, the laccase activity estimated was much higher than in the original culture filtrate. This is in concordance with Lou *et al.*, (2005) who have experimentally proven that inhibition by sea water is reversible, once the inhibiting ions have been removed.

Another *Cerrena* strain; *Cerrena unicolor* strain 137 was reported to produce 18,700 U L⁻¹ (Michniewicz *et al.*, 2006). Our strain produces a higher titer of laccase than this strain (Gianfreda *et al.*, 1998; Michniewicz *et al.*, 2006; Stepanova *et al.*, 2002) even in the presence of inhibiting salts of seawater. Isolates of *Pycnoporus* from various geographical regions of China were reported to produce lower titers of laccase (11,000 - 17,000 U L⁻¹) (Lomascolo *et al.*, 2002). Thus, *Cerrena unicolor* MTCC 5159 can be acknowledged as marine-derived laccase hyper-producing fungal strain.

For the yield experiment, EPS was not removed since its removal results in great loss of laccase activity due to the entrapment of laccase in the EPS matrix and the subsequent freezing and thawing steps required to remove it. Even though several molecular mass and surface charge laccase isozymes were present in the partially purified culture filtrate, a 33-fold purification with a final yield of 17 % of a single isoform (Lac IId) was achieved. In comparison, *C. unicolor* strain 137 which was reported to produce Lacc I and Lacc II, which together gave a total yield of 22 % (Michniewicz *et al.*, 2006), on purification.

Laccases have characteristic spectral properties. Lac IId exhibited a peak at 610 nm, typical for the T1 Cu responsible for the blue colour of the enzyme, a characteristic typical of blue laccases. It also exhibited a shoulder at 330 nm which suggests the presence of the T3 binuclear Cu pair, which forms a part of the tri-nuclear cluster T2 Cu.

Comparison of N-terminal sequence of Lac IId with other N-terminal sequences showed 70 - 85 % similarity with basidiomycetous laccases. Maximum similarity was observed with laccases from the white-rot basidiomycetes *Schizophyllum commune* and *Spongipellis* sp. Even though the N-terminal sequence of *Volvariella* did not have very high homology to the N-terminal of Lac IId it was still included in Table 3 since an internal peptide of a *Volvariella* laccase had maximum homology with an internal peptide of the Lac IId.

The internal peptide of 17 AAs, (generated by trypsin) from Lac IId demonstrated complete homology to an internal peptide (Q69FX1_9AGAR) of 'Laccase 2' of *Volvariella volvacea* which had a predicted molecular mass and pI of 59367 Da (~59 kDa) and 5.4, respectively. This was almost identical to the experimentally determined molecular mass and pI of Lac IId which was 59 kDa and 5.3 respectively, further endorsing the results.

The inability to identify more peptides may have been due to the HCCA matrix used instead of a matrix that is specific for glycoproteins such as 2, 5-dihydroxybenzoic acid (DHB), super DHB (sDHB), 2,4,6-trihydroxyacetophenone (THAP) or DHAP. It could also be due to the novelty of this laccase considering its marine source and that the current MALDI database is not comprehensive enough to account for it since not much work has been done for marine-derived laccases, in terms of sequence determination.

Laccase from *Pycnoporus sanguineus* (SCC 108) had an optimum temperature at only 55°C (Litthauer *et al.*, 2007). Whereas using ABTS as substrate, partially purified laccase had a bimodal pH optima at 3 and 6 and a temperature optima at 60°C, the bimodal pH profile indicates the presence of at least 2 laccase isozymes as observed in Fig. 4. For pure Lac IId, the pH and temperature optimum was at 3 and 70°C, respectively. This is consistent with data that pH optima for the oxidation of ABTS are generally lower than 4 (Baldrian, 2006).

Lac IId had pH optima at 6 when syringaldazine and guaiacol were used as substrate. This is in concordance with known higher pH optima values for phenolic compounds which range between 4 and 7 (Baldrian, 2006). The pH optima of laccases are highly dependant on the type of substrate. If the substrate is phenolic in nature, the pH profile is bell-shaped, which is formed by two opposing effects. The oxidation of phenols

depends on the redox potential difference between the phenolic compound and the T1 copper (Xu, 1996). The E° of a phenol decreases when pH increases due to the oxidative proton release. At a rate of $\Delta E / \Delta \text{pH} = 59 \text{ mV}$ at 25°C i.e. a pH change from 2.7 to 11 would result in a 490 mV decrease in E° of the phenol. However, over the same pH range, the E° decrease for the fungal laccases studied (*T. villosa*, *R. solani* and *M. thermophila*) were much smaller ($\leq 100 \text{ mV}$) (Xu, 1997). The enzyme activity at higher pH, decreases due to the binding of a hydroxide anion to the trinuclear coppers of laccase. This interrupts the internal electron transfer from T1 to trinuclear centre (Munoz *et al.*, 1997). Not only the rate of oxidation but also the reaction products may differ with varying pH since pH may affect abiotic follow-up reactions of primary radicals resulting from laccase catalysis. Laccases from *Rhizoctonia praticola* and *T. versicolor* catalyzed the formation of different products from syringic and vanillic acids at varying pH values but both enzymes generated the same products at a particular pH (Xu, 1997).

ABTS is electron-rich like phenolic compounds such as guaiacol or syringaldazine but is not phenolic in nature. Hence the oxidation potential of ABTS is not pH-dependent within the range 2 – 11 and proceeds in a single step (Terrón *et al.*, 2004). Hence we can conclude that Lac IId has optimum activity at pH 3 excluding interference from the substrate.

It is common for basidiomycete laccases 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 was most stable at pH 9 where it showed no activity. Similar results were obtained with laccase of *Peniophora* sp. (Niku-Paavola *et al.*, 2004) and *C. unicolor* strain 137 (Michniewicz *et al.*, 2006). Lac IId retained 67 % of its activity at 4°C for over 35 days and 100 % activity when stored at -20°C for over 1 year. This makes it a potential commercial candidate.

Lac IId's half-life of 90 min at 70°C is much superior to other laccases such as, Lacc I from *C. unicolor* strain 137 which completely lost its activity after less than 10 min at 70°C and Lacc II which had a half life of only 10 min at 70°C (Michniewicz *et al.*, 2006). Laccase from *Trametes* was reported to have a half-life of 27 min at 75°C (Xiao *et al.*, 2003) while the laccase from an ascomycete, *Mauginiella* displayed a half-life of 3 min at the same temperature (Palonen *et al.*, 2003). Thermostable laccase from *Peniophora* sp. displayed a half-life of 15 min at 75°C (Niku-Paavola *et al.*, 2004),

whereas [Jordaan and Leukes \(2003\)](#) reported a fully active laccase at 60°C for 9 h from an unidentified basidiomycete. In terms of thermostability, Lac IId occupies a superior position.

The Q_{10} values were found to be >1 between 20° and 70°C in the pH range of 3 - 7 for Lac IId ([Table 5](#)), indicating its temperature dependency for activity in this pH and temperature range. In contrast, the Q_{10} values <1 for temperatures above 70°C suggest the negative effect of these temperatures on the enzyme activity. The Q_{10} value of above 1 between 20° – 70°C also lends support to the conclusion that Lac IId is a high-temperature active enzyme.

The energy of activation (E_{act}) for Lac IId between 60° and 70°C at its optimum pH (3) was 2.5 kJ mol⁻¹. This was lower than 3.9 kJ mol⁻¹ of *Pleurotus florida* laccase ([Das et al., 2001](#)) and 12.4 kJ mol⁻¹ of *P. sajor-caju* laccase ([Lo et al., 2001](#)) besides the temperature optima for these laccases were much lower at 50° and 45°C, respectively. The temperature profile of Lac IId was more of plateau for a wide range of temperature, indicating that its activity was not adversely affected by high temperature. This, along with the high turnover numbers for oxidation of ABTS by Lac IId at 70°C (K_{cat} and K_{cat}/K_m values) further attest its thermostable character.

Some low molecular weight compounds that can be oxidized to stable radicals by laccase, can act as redox mediators oxidizing other compounds that in principle are not substrates of laccase. In addition, to enable the oxidation of non-substrates e.g. the nonphenolic lignin moiety, the mediators can diffuse far away to sites that are inaccessible to the fungus of the enzyme itself. When ABTS was used as substrate, the activity of Lac IId increased with four of the known mediators (hydroquinone, 3, 5-dimethoxy-4-hydroxyacetophenone, syringic acid and veratryl alcohol), confirming their role as mediators. Lac IId was non-responsive in the presence of 4-hydroxy-2, 2, 6, 6-tetra-methylpiperidin-1-oxyl. The combination of 1-hydroxybenzotriazole and ABTS proved to be antagonistic resulting in an overall decrease in laccase activity.

Although ABTS does not occur naturally in nature, most laccases have great affinity for it as a substrate. Among the substrates tested with Lac IId, ABTS was found to be the best followed by ferulic acid and guaiacol. Whereas tyrosine, vanillic acid, 2, 5-dimethyl aniline, *p*-anisidine and violuric acid were not oxidized by Lac IId. Tyrosine

was used as a negative control, since a true laccase should be unable to catalyze the oxidation of tyrosine. Besides acting as a substrate, ABTS is also known to act as a mediator as well when another compound was used as substrate. In the presence of ABTS, most of the compounds got oxidized which, previously did not get oxidized (with the exception for vanillic acid and tyrosine). This indicates the mediating role of ABTS in the oxidation of these compounds when exposed to Lac IId.

Laccases can be inhibited when the inhibitor binds strongly to it stopping further catalysis of the reaction. This occurs when the Cu at the catalytic center is removed / chelated or by competing for O₂, which is the specific co-substrate of laccase. Laccases have been known to be inhibited by diethyl dithiocarbamate and thioglycolic acid probably due to their effect on copper at the catalytic centre of laccase and by several sulfhydryl compounds such as dithiothreitol, thioglycolic acid, cysteine and diethyldithiocarbamic acid (Baldrian, 2006). This is true for laccase from *P. cinnabarinus* which was totally inhibited by 1 mM L-cysteine and DTT (Eggert *et al.*, 1996a). However, Lac IId from *Cerrena unicolor* MTCC 5159 was neither inhibited in the presence of 0.1 and 1 mM L-cysteine nor with 1 mM DTT. Experiments with *T. versicolor* laccase showed that the inhibitory effect found with these compounds does not occur in the same way as inhibition by azide which decreases the oxygen consumption by laccase during the catalysis by competing for its essential co-substrate which is molecular oxygen (Johannes & Majcherczyk, 2000).

In presence of 4-hexyl resorcinol, Lac IId showed activity, this was because it is a known inhibitor of only tyrosinase and not laccase (Dawley & Flurkey, 1993). However, CTAB, a known inhibitor of laccase was able to inhibit Lac IId by only ~30 %. The broad range of substrates accepted by laccase as hydrogen donors, oxidation of syringaldazine in combination with the inability to oxidize tyrosine and ability to remain active in the presence of 4-hexyl-resorcinol, has been taken to be an indicator of laccase activity (Thurston, 1994). Unambiguous determination of laccase activity was best achieved by purification of the protein to electrophoretic homogeneity followed by determination of K_m with multiple substrates which include substrates such as syringaldazine and ABTS, for which laccase has a high affinity, and tyrosine for which laccase has no affinity (Edens *et al.*, 1999; Shin & Lee, 2000) as seen with Lac IId.

Heavy metals are reported to have a significant negative effect on the action of laccases in general (Baldrian, 2003), however Lac IId doesn't comply with this. Only chromium and tungsten were able to maximally inhibit it by 56 and 48 % respectively, whereas the other metals showed even lesser inhibition.

The wide spectrum of metal ions that Lac IId is resistant to, suggests that it is metal-tolerant and has a great bioremediation potential in heavy metal contaminated environments, even though a heavy metal like Cu is a well known inducer for laccase (Baldrian, 2003; Galhaup & Haltrich, 2001). For all the metals tested, chloride was present as the chloride salt of the metal. Thus, whether the inhibitory effect of the metal salt was due to the combined effect of the metal and the chloride or due to the metal alone, could be ascertained by testing the activity of Lac IId in the presence of ammonium chloride. Ammonium, not a heavy metal was used in the medium optimization studies for laccase production. Ammonium was used as its chloride salt. Here the effect of chloride could be tested independently, if any inhibition had to occur, it would have to be due to chloride alone. As seen in Table 11, chloride shows no inhibition, since its electronegativity is lower than that of oxygen (co-substrate of laccase), it probably cannot competitively inhibit it. In addition, the amount of chloride tested was lower than which is present in seawater.

The Lac IId from *Cerrena unicolor* MTCC 5159 has all the characteristics of a typical blue laccase: (i) blue colour due to its absorbance at 610 nm, (ii) a shoulder at 330 nm which is representative of T3 binuclear copper pair, (iii) N-terminal amino acid sequence showing 70 – 85 % homology to other basidiomycete laccases, (iv) Internal peptide showing 100 % homology to *Volvariella volvacea* (basidiomycete) laccase, (v) its inability to oxidize tyrosine and high affinity for syringaldazine and ABTS and (vi) its non inhibition by 4-hexyl-resorcinol, a selective inhibitor of tyrosinase.

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 sea water, indicating its salt tolerance, makes the marine-derived *Cerrena unicolor* MTCC 5159 a suitable candidate for bioremediation.