ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 5 (4): 522-529, 2006
ISSN 1682-296X

Isolation, Molecular Characterization and Reactivity with 2,6 Dichlorophenol of a Laccase and Isolation of Laccase Gene Specific Sequences from Lignin Degrading Basidiomycete *Phanerochaete chrysosporium* (TL 1)

P.C. Prabu, C. Udayasoorian and G. Balasubramanian
Department of Environmental Sciences, Tamil Nadu Agricultural University,
Coimbatore-3, Tamil Nadu, India

Abstract: A new lignin degrading basidiomycete, *Phanerochaete chrysosporium* (TL 1) was isolated from pulp and paper mill effluent enriched soil samples can be induced to produce high level laccases when grown on a cellobiose-asparagine liquid medium with 150 μM $CuSO_4$. The fungus grown under static conditions had 70% of total extra cellular laccase protein and about 2.5 fold purification with a final yield of 13.2% of protein purification by Sephadex G-100 column and FPLC. The resultant enzyme pool of the purification process is found to contain a single polypeptide, which produced a single band on an SDS-PAGE. The purified protein showed a specific activity of 106 U mg^{-1} and the molecular mass (Mr) of native laccase was 65 kDa. The purified laccase has an isoelctric point of 4.0, it is stable in pH range from 4.0 to 6.0 and its optimum pH is 4.5. The optimal reaction temperature is 60°C and stable at 70°C for more than 1 h. Degenerative primers corresponding to the consensus sequences of the copper binding regions in the N-terminal domains of known basidiomycete laccase were used to isolate laccase gene specific sequences from this strain and the laccase gene gave PCR product of about 150 bp and cloned product gave 85% similarity with laccase from *T. villosa* LCC 2 (L49377).

Key words: Laccase enzyme, laccase gene, *Phanerochaete chrysosporium* (TL 1)

INTRODUCTION

Lignin is an aromatic heteropolymer of phenyl-propanoid units which confers structural rigidity to woody plant tissues and protects them from microbial attack (Higuchi, 1990). White rot fungi efficiently degrade lignin, a complex aromatic polymer in wood that is among the most abundant natural materials on earth (Martinez *et al*., 2004). It is well established that the white rot fungi are most effective in lignin degradation in nature, synthesizing a ligninolytic system including lignin peroxidase (Lip), manganese peroxidase (Mnp) and laccase, which are responsible for the degradation of lignin. Most white-rot fungi capable of degrading lignin can synthesis Mnp and laccase. Actually, the combination of Mnp and laccase is much more common than that of Lip and Mnp. However, the white-rot basidiomycete *Coriolopsis rigida* secretes no detectable Lip or Mnp but enough extra cellular laccase (Saparrat *et al*., 2002).

In the presence of appropriate redox mediators, such as 2,2'-azino-bis (3-ethylbenzothazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT), laccase also catalyzes the oxidation of non-phenolic lignin model compounds (Bourbonnais and Paice, 1990) and degrades polycyclic aromatic hydrocarbons (Pickard *et al*., 1999) and various dye pollutants. Because of the significance of potential applications in biopulping, kraft lignin bleaching or degradation of aromatic pollutants and wastewater treatment, laccase draws considerable attention from researchers. Many laccases have been purified and characterized (Min *et al*., 2001; Xiao *et al*., 2003). However, the mechanism of fungal degradation of lignin by laccase remains to be defined. Therefore, purification and characterization of laccase from novel white-rot fungi will help shed light on this mechanism.

The white-rot fungi *Phanerochaete chrysosporium* (TL 1) isolated from enriched pulp and paper mill effluent irrigated soil samples for the past 20 years was able to selectively degrade lignin compounds, phenols and for wastewater treatment. In order to investigate the ligninolytic system of *Phanerochaete chrysosporium* (TL 1) and the role of its laccase in lignin degradation, laccase was purified and characterized and its reactivity with 2, 6 dichlorophenol was studied. The isolation of laccase gene specific sequences was carried out for further exploitation of its phenol degradation potential, wastewater treatment and other industrial applications.

Corresponding Author: P.C. Prabu, Department of Environmental Sciences, Tamil Nadu Agricultural University, Coimbatore-3, Tamil Nadu, India

MATERIALS AND METHODS

Fungal strain: The microorganism was isolated from enriched soil samples with 20 years of continuous effluent irrigation by employing standard serial dilution plating technique at bioremediation laboratory of Tamil Nadu Agricultural University, Coimbatore during 2003. The isolated fungus was screened for its ligninolytic activity based on the growth on media containing phenol red. Plates were observed for fungal growth and colour change in the culture media from yellow to red around the colonies, which indicate the ligninolytic nature of the culture. Lignin degradation by the isolated fungal culture was confirmed by quantifying the $^{14}CO_2$ produced during the metabolism of ^{14}C labeled synthetic lignin obtained by polymerization of labeled coniferyl alcohol (Kirk *et al.*, 1975). The screened fungal culture was grown at 30°C for 5 days on the CPDA medium (20.0 g glucose, 1.0 g KH_2PO_3, 1.5 g $MgSO_4.7H_2O$, 50 μg vitamin B1, 15.0 g agar powder and 1,000 mL potato extract liquid (20%)) and the pure strain was stored at 4°C in CPDA slants and inoculated once in every 3 months.

Growth conditions and induction of enzyme production: For studies on enzyme production, 5-6 cylinders (diameter, 10 mm) of this strain grown on CDPA plates were used to inoculate 250 mL Erlenmeyer flasks containing 100 mL liquid cellobiose asparagines medium. The liquid culture medium contained (per litre): 10 g cellobiose, 10 mL glycerol, 1.5 g L-asparagine, 1.0 g KH_2PO_3, 0.5 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2$, 0.001 g $FeSO_4.7H_2O$, 0.1 g $Na_2HPO_4.5H_2O$, 0.002 g $CuSO_4.5H_2O$, 0.0275 g adenine and 50 μg vitamin B1. The culture was incubated at 30°C on a rotary shaker at 140 rpm. A 72 h old liquid culture was homogenized using a sterilized blender. The volume of inoculum was 10 mL per 200 mL culture medium, which was then cultivated under the same conditions and the extracellular ligninolytic enzyme production was estimated on 3rd, 5th, 7th 9th and 11th day. The addition of 150 μM $CuSO_4$ on the third day of incubation was assayed as an inducer of laccase activity. Samples were taken periodically from four replicate flasks and the mycelium was separated from the culture liquid by centrifugation at 20000 x g and 4°C for 10 min.

Analysis of protein, reducing sugars and enzymatic assay: Extracellular protein was determined by Bradford method by using Bio-Rad protein assay and bovine serum albumin as the standard. Reducing sugars were assayed by Somogyi and Nelson method, with glucose as the standard. Laccase (p-diphenol oxygen oxidoreductase; E.C. 1.10.3.2) assay was performed based on monitoring the rate of oxidation of syringaldazine (Sealey and Ragauskas, 1998) and plate assay of laccase activity was done as per the method of Srinivasan *et al.* (1995).

Separation and purification of laccase proteins: *Phanerochaete chrysosporium* (TL 1) laccase was purified from 9 day old cultures containing $CuSO_4$. The laccase enzyme proteins were separated and fractionated by Sephadex G.100 column and further purified by Fast Performance Liquid Chromatography (FPLC) using superpose at the rate of 6 column and SDS –PAGE (Bollag *et al.*, 1996).

Isoelectric point and copper content: Analytical isoelectric focusing PAGE was performed with a mini isoelectric focusing cell (Bio rad) with a pH gradient of 2.5 to 50. the copper content of purified enzyme was determined by atomic absorption spectrometry.

Enzyme characterization: Estimates of the laccase optimum pH were obtained by using 50 mM acetate buffer (3.6 to 5.5) and 50 mM citrate phosphate buffer (pH 2.6 to 7) and the pH stability was assayed in the pH range of 2.0 to 6 using 50 mM citrate phosphate borate buffer. The optimum temperature and stability was determined between 50 to 80°C using citrate buffer.

Standardization of laccase assay: Laccase assay was standardized and performed with different substrates viz., guaiacol, 2,6 dimethoxy phenol (DMP), ABTS (2,2'-azinobis-3ethylbenzthiazoline-6-sulfonate) and standardized. This was qualitatively explored by changes in the optical absorbance spectra of the reaction mixtures which contained 0.5 mM of ABTS (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M sodium acetate buffer (pH 6.0). On addition of enzyme source oxidation of ABTS was monitored by determining the increase in A_{420} (A_{420} = $3.6x10^4$ M^{-1} cm^{-1}), guaiacol (A_{465} = $1.2x10^4$ M^{-1} cm^{-1}) and DMP (A_{468} = $4.96x10^4$ M^{-1} cm^{-1}) in a spectrophotometer (EC India Ltd.,). Heat killed enzyme served as the control. One unit of enzyme activity is defined in μmol of ABTS oxidized min^{-1}. Kinetic studies were performed at 25°C by measuring the initial velocity in 3 mL glass cuvettes with 1 cm path length Inhibitor studies were carried out using guaiacol in 10 mM citrate-Na_2HPO_4 buffer (pH 6.0).

HPLC analysis of 2,6 dichlorophenol: 2,6 dichlorophenol was used as substrate for testing the oxidation activity of laccase. The reaction mixture (1.0 mL) contained 100 μM substrate, 1 mM ABTS and 2.0 U laccase in 0.1 M acetate buffer pH 5.0 at 25°C. Two controls without ABTS and laccase were also used. Samples were collected after

0, 6, 12 and 18 h. Twenty microliter of each sample was injected in to the HPLC system equipped with a reversed phase C18 column. Substrate was monitored by UV absorbance at 254 nm and oxidation was calculated from reduction in peak area and compared to a standard curve.

Extraction of genomic DNA: The Extraction of genomic DNA was carried out as per the method of Gawel and Jarret (1991). The purified and diluted (25 ng μL^{-1}) DNA sample was used for PCR amplification. The forward (5' CAT TGG CAT GGN TTT TTT CA 3') and reverse primer (5' ATG GCT GTG GTA CCA AAA NGT 3') for the amplification of the specific laccase gene loci were obtained from the results of D'Souza *et al.* (1996).

Cloning and sequencing of PCR amplified products: DNA bands corresponding to the major PCR amplified products were sliced out of the agarose gels and the DNA was purified. Selected PCR amplified products were cloned in to a T vector system devised as described by Marchuk *et al.* (1991). Plasmid DNA containing the cloned PCR amplified products from *E. coli* cells was extracted and purified from 5 mL cultures. Cloned PCR amplified products were then sequenced with T3 and T7 fluorescent primers at the department of Biotechnology, Madurai Kamaraj University, Madurai. Nucleotide similarities and translation of the exon sequences were done with the GENEPRO program.

RESULTS

Isolation and culture of ligninolytic microorganism: The isolated microorganism identified as *Phanerochaete chrysosporium* (TL 1) based on colony, cell morphology, physiological and biochemical characteristics and verified at Mycology Department of Indian Agricultural Research Institute (IARI), New Delhi. The lignin degrading ability of the fungus was also confirmed by measuring the quantity of $^{14}CO_2$ evolved from degradation of ^{14}C DHP synthetic lignin (Fig. 1). The fungus released 33.6% of $^{14}CO_2$ and the per cent utilization of synthetic lignin for *Phanerochaete chrysosporium* was 44.3 and fixed as cell carbon.

Production of extracellular enzymes, reducing sugars and proteins: We monitored the extracellular enzymes activity, protein and reducing sugars in *Phanerochaete chrysosporium* (TL 1) culture for 11 days. Maximum laccase activity was observed at 9 days of growth. The addition of Cu^{2+} in the culture medium enhanced the laccase activity compared to no addition (Fig. 2). Zymograms of laccase after isoelectric focusing of crude enzyme preparations obtained from cultures carried out in the absence or presence of Cu^{2+} resulted in a single band with a pI of 4. The activity band from Cu^{2+} induced cultures corresponded to the major protein band, seen after staining the gel with coomassie blue. The optimal pH of laccase activity in crude enzyme preparation from Cu^{2+} induced cultures was 4. Laccase was stable from pH 5 to 6 at room temperature for 24 h, retaining 50 and 40% activity at pH 5 and 4, respectively (Data not shown). The production of extracellular laccase by *Phanerochaete chrysosporium* (TL 1) was assayed on agarose plates containing ABTS and the development of an intense bluish green color showed that the presence of laccase activity in the extracellular fluid of *Phanerochaete chrysosporium* (TL 1) (Fig. 3).

Separation and purification of laccase protein from *Phanerochaete chrysosporium* (TL 1): The *Phanerochaetes chrysosporium* grown under static conditions was harvested on day nine, at peak laccase

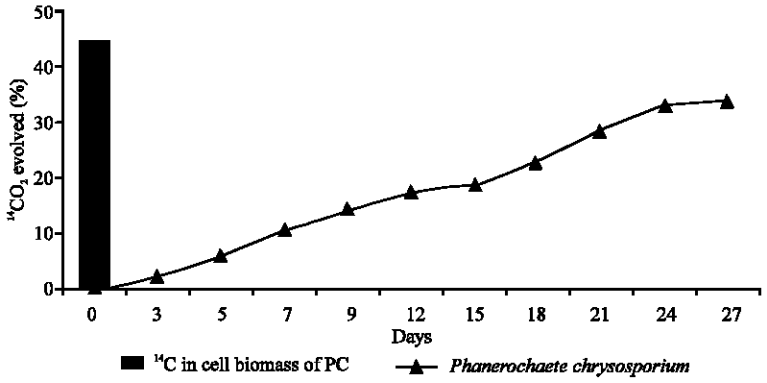


Fig. 1: Degradation of ^{14} C DHP synthetic lignin by *Phanerochaete chrysosporium* (TL 1)

Table 1: Purification of extracellular laccase from *Phanerochaete chrysosporium* (TL 1)

Purification step	Volume (mL)	Protein (mg L^{-1})	Total activity (U L^{-1})	Yield (%)	Specific activity (U mg^{-1})	Purification factor (fold)
Culture filterate	2015	112.0	4700	100	42	0.00
Sephadex G - 100	6.00	48.0	3600	76	75	1.80
Superose 6	1.50	13.2	1400	28	106	2.50

Laccase was purified from 2L culture grown under stationary culture conditions, Laccase activity was determined with 5 mM as substrate; expressed as µmol of ABTS oxidized per minute

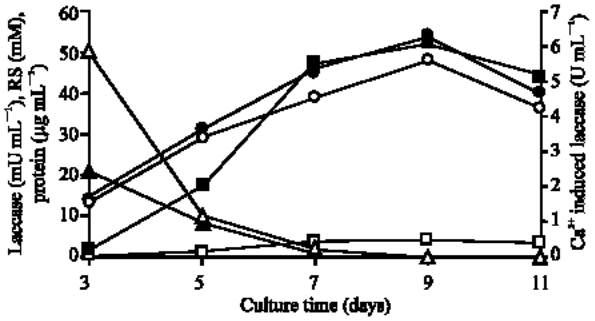


Fig. 2: Time course of laccase activity (□), protein (○) and reducing sugars (Δ) in the extra cellular fluid of *Phanerochaete chrysosporium* (TL 1) grown in absence and presence of copper (open and closed symbols, respectively)

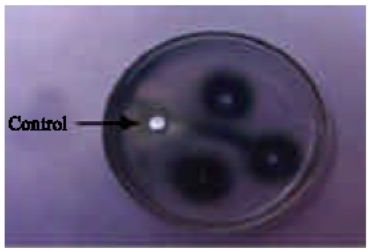


Fig. 3: Plate assay for laccase activity by *Phanerochaete chrysosporium* (TL 1)

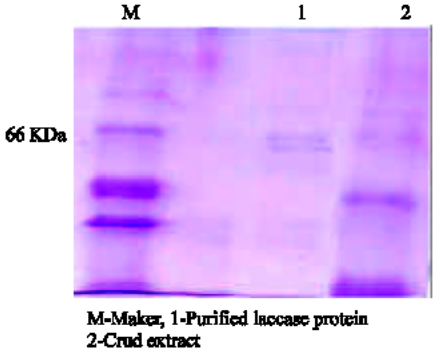


Fig. 4: Purified laccase protein of *Phanerochaete chrysosporium* (TL 1)

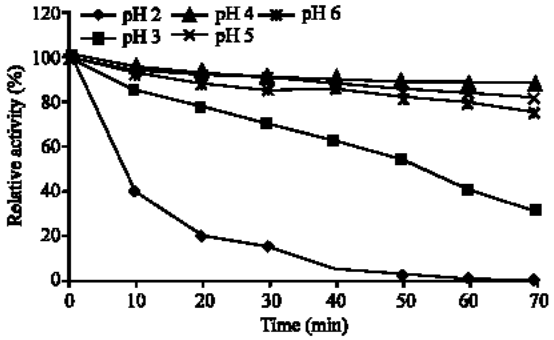


Fig. 5: Purified laccase activity at different pH

activity and accounted for about 70% of the total extracellular protein. The culture broth was contaminated by pigments or coloured, which were largely removed by initial freeze-thaw and protein precipitation with ammonium sulphate. The laccase was further purified to apparent homogeneity according to the procedure summarized in Table 1. During the first step, extra cellular culture fluid was applied to Sephadex G-100 (Pharmacia, Uppasala, Sweden) column, in which the third fraction showed maximum laccase activity. The fractions showing laccase activity were pooled and further purification of protein accomplished by Fast Performance Liquid Chromatography (Pharmacia, Uppasala, Sweden) using a Superose 6™ column. The laccase activity eluted as a single peak and about five fold purification with a final yield of 13.2% of protein purification was achieved. The purified protein showed a specific activity of 106 U mg^{-1}.

As determined by calibrated gel filtration chromatography on Superose 6, the molecular mass (Mr) of native laccase was estimated to be 65 kDa (Fig. 4).

Separation of laccase proteins by SDS-PAGE: The extra cellular culture fluid of *Phanerochaete chrysosporium* (TL 1) contained six electrophoretically separable proteins, of which one protein exhibited laccase activity. The resultant enzyme pool of the purification process described earlier was found to contain a single polypeptide, which produced a single band on an SDS-PAGE. The molecular mass (Mr) of purified laccase protein was estimated to be 65 kDa. These results suggest that the enzyme is monomeric. The isoelectric point of laccase to be 4.0, which suggests it is an acidic protein.

Effects of pH and temperature: The effect of pH and temperature on the activity of laccase showed a typical

Table 2: Kinetic constants of laccase from *Phanerochaete chrysosporium* (TL 1)

Substrate	K_m (μM)	V_{max} (μM. min^{-1} mg^{-1})	k_{cat} (S^{-1})	k_{cat} (S^{-1})/ K_m (S^{-1} M^{-1})
ABTS(2,2'-Azino-bis (3 ethyl-benzothazoline -6 –sulfonate)	21.0	525.0	551.0	$26.2x10^6$
Dimethoxy phenol	22.0	66.0	68.0	$3.1x10^6$
Guaiacol	398.0	57.0	59.0	$1.5x10^5$

Table 3: Effect of inhibitors and organic solvents on the oxidation of guaiacol by laccase

	Inhibition (%)					
Concentration (mM)	Sodium azide	Cyanide	SDS	TFA	DMSO	EDTA
Control	0	0	0.0	0.0	0.0	0.0
0.1	100	98	5.5	0.0	0.0	0.0
0.5	100	99	6.0	7.9	0.0	7.9
1.0	100	100	8.0	10.6	0.0	10.5
5.0	100	100	38.1	13.1	2.1	13.1
10.0	100	100	50.2	24.8	3.0	24.5
25.0	100	100	62.7	42.5	3.7	42.0

SDS- ; DMSO- Dimethylsulfoxide; TFA- Trifluroacetic acid

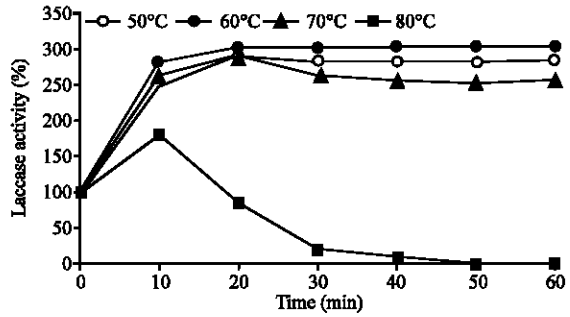
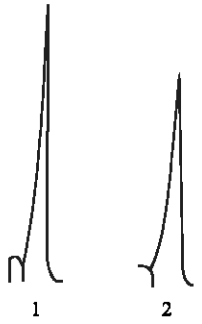


Fig. 6: Purified laccase activity at different temperature



1. 6 h after incubation; 2. 12 h after incubation; 18 h-complete degradation

Fig. 7: Degradation of 2,6-dichlorophenol by purified laccase of *Phanerochaete chrysosporium* as detected by HPLC. Peaks 1 and 2 were detected after 6 and 12 h incubation. At 18 h complete degradation occur

bell curve. The optimum pH of the enzyme was 4.5 in citrate - Na_2HPO_4 buffer for guaiacol and the enzyme was stable from pH 4.0 to pH 6.0 (Fig. 5). The temperature optimum was 60°C and the enzyme was stable at 70°C for more than 1 h (Fig. 6).

Substrate specificity: The assay of laccase was standardized with the use of different substrates viz., syringaldazine, ABTS and guaiacol. It was found that a reaction mixture containing 0.5 mM of ABTS in 0.1 M sodium acetate buffer (pH 6.0) along with the enzyme source could be used as a standard laccase activity assay and one unit of enzyme activity was defined as μmol of ABTS oxidized per min and the laccase exhibited high activity with ABTS (Table 2). The apparent K_m value of the enzyme for ABTS determined from the Lineweaver-Burk plot was estimated to be 21 μM. The apparent K_m values determined for syringaldazine and guaiacol were 22 and 398 μM, respectively. The k_{cat} values for ABTS, 525 S^{-1}, was higher than that of syringaldazine or guaiacol. This result suggested that ABTS should be an effective substrate of this enzyme. Catalytic efficiencies of laccase k_{cat}/k_m for the substrates varied from $1.50x10^5$ for guaiacol to $26.2x10^6$ for ABTS.

Inhibitor and organic solvent studies: The effects of several potential inhibitors on laccase activity were examined with guaiacol as a substrate at pH 4.5 (Table 3). The laccase activity was totally inhibited by 0.1 mM sodium azide or cyanide, 62.5% inhibited by 25 mM SDS and almost unaffected by 25 mM EDTA. Activity of of laccase when analysed on organic solvent solution was stable to some extent in trifluoroacetic acid (TFA). Laccase was almost completely stable and efficient in 25 mM dimethylsulfoxide (DMSO).

2,6-Dichlorophenol degradation: 2,6-Dichlorophenol was degraded by purified laccase in the presence or absence of ABTS. Two units of purified laccase alone was able to completely oxidize 100 μmol 2,6-dichlorophenol in 18 h (Fig. 7).

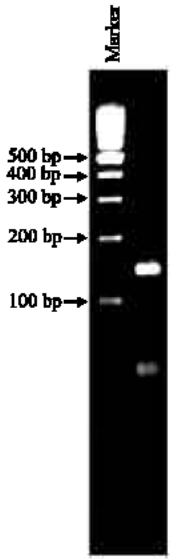


Fig. 8: Amplified laccase gene products

Isolation and amplification of laccase specific gene: Fungal DNA was isolated by CTAB extraction method and the quantity and quality of DNA was tested by fluorometry and agarose gel electrophoresis so as to ensure the use of good quality DNA for PCR analysis. The quantity of DNA in sample was varied from 1500-2500 ng μL^{-1}. This variation was due to several reasons such as difference in the sample size taken for DNA extraction and pipetting errors due to various contamination during DNA extraction procedure. After quantification the samples were diluted to 25 ng μL^{-1} and it was used for PCR reactions. Gene specific forward and reverse primers were used in PCR amplification and both the samples amplified specific ~150 bp PCR product by amplifying the template DNA with *Taq* polymerase (Bangalore Genei Pvt., Ltd., Bangalore, India). The amplified products were separated on 1.5% agarose gel and stained with ethidium bromide. They were viewed and documented (Fig. 8). The amplified laccase gene was cloned and sequenced and had identical sequences. Comparison of nucleotide sequences revealed 85% similarity with laccase from *Trametes villosa* LCC 2 (L49377) and *Pycnoporus cinnabarinus* (not shown) Lac 1 gene (AF 170093), respectively.

DISCUSSION

Lignin is the single most important activity in the biological cycle of carbon. The multitude of interunit bonds and functional groups and the heterogeneity of the polymer is the main reason for the resistance of lignin to microbial attack and it is in fact one of the most recalcitrant naturally occurring biological material. Considerable attention has been focused on the function of extracellular laccase in the decomposition of lignin. Apart from lignin biodegradation, laccase finds its way in industrial applications and environmental safety. White-rot and brown-rot fungi play different role in lignin biodegradation.

In the present study, we describe the isolation of a lignin degrading fungus from the soil enriched by irrigation over 20 years by paper mill effluent. This fungus is a whiterot basidiomycete and able to oxidize phenol red and ^{14}C synthetic lignin. It is unequivocally accepted that the ^{14}C DHP mineralization to $^{14}CO_2$ is considered as the confirmation of ligninolytic activity of the organism and it convincingly demonstrate the rate and extent of lignin degradation (D'Souza *et al*., 1999). The non-specific oxidation caused by enzymatic combustion leads to the formation of CO_2. The production of laccase by whiterot fungi is widespread and is the sole ligninolytic enzyme produced by some basidiomycete, *Pycnoporus sanguineus* (Ponting *et al*., 2000) and *Coriolopsis gallica* (Calvo *et al*., 1995). Laccase synthesis is induced by many kinds of phenolic compounds and substrate analogs, and the optimum induce varies from one strain to another. For instance, the most suitable inducer for laccase synthesis by white rot fungus *P. cinnabarinus* ss3 is ferrulic acid, but in the case of *C. hirsutus*, it is syringaldazine (Herpoel *et al*., 2000) and for *Trametes* sp. AH28-2 it is kraft lignin (Xiao *et al*., 2003). Copper induces laccase at the level of gene transcription in *Coriolopsis rigida* (Saparrat *et al*., 2002), and we found a similar effect in *Phanerochaete chrysosporium* (TL 1) (Fig. 2). The laccase enzyme detected in fungal culture supernatants upon addition of Cu was purified to electrophoretic homogeneity.

Compared with other fungal laccases, the characteristics of purified *Phanerochaete chrysosporium* (TL 1) laccases are typical. Based on the contour of conditions for laccase production, the culture *Phanerochaete chrysosporium* (TL 1) was mass multiplied and the protein was separated by gel filtration chromatography and the protein profile studied and characterized. Molecular weight of most fungal laccase proteins fall between 43 to 110 kDa (Yoropolov *et al*., 1994) and a majority of laccases from basidiomycetes fungi were reported to have molecular weights in the range of 55 to 72 kDa. The *Phanerochaete chrysosporium* (TL 1) laccase protein was found to have a molecular weight of 65 kDa. The *Phanerochaete chrysosporium* (TL 1) laccase has high substrate specificity and able to oxidize phenol red. The fungal laccase belong to the class of the blue oxidases that generally contain four copper

atoms per molecule and in accordance with Das *et al*., (2001). The purified *Phanerochaete chrysosporium* (TL 1) laccase showed quite low stability below pH 3.5, but high stability in weak acid, neutral and weak alkali environments and supported by Xiao *et al*. (2003). The enzyme is stable at 70°C for more than 1 h (>90% activity remained). At 75°C, the half life of laccase is 30 min. These findings indicate that purified laccase has good thermal stability which is similar to that of one of the most heat resistance laccase from *Trametes* sp. Strain AH28-2 (Xiao *et al*., 2003). As activity at high pH and temperature is usually required for industrial applications the purified laccase from *Phanerochaete chrysosporium* (TL 1) may be a good target for the development of biotechnological tools.

The ability of laccase to catalyze 2,6 dichlorophenol one among the group of chlorinated phenols considered one of the most dangerous environmental contaminants. Under experimental conditions purified laccase is able to convert 2,6 dichlorophenol directly. This result is in agreement with the investigations by Xiao *et al*. (2003).

PCR analysis of laccase gene and sequencing of laccase gene: Laccases are copper containing oxidases, which catalyze the four electron oxidation of a variety of phenolic compounds and a simultaneous four electron reduction of oxygen to water. The PCR strategy used in this study is based on the use of degenerative primers corresponding to the consensus sequences conserved in the copper binding regions in the N terminal domains of known basidiomycete laccases. The quantity of DNA in the fungal isolates varied from 1500-2500 ng μL^{-1}. After quantification the samples were diluted to 25 ng μL^{-1} and it was used for PCR reactions. Gene specific forward and reverse primers were used in PCR amplification and the fungus samples amplified specific ~150 bp PCR product by amplifying the template DNA with *Taq* polymerase. This is in line with the findings Xiao *et al*. (2003), who reported that the size of the PCR product varied from ~ 144 to 200 bp. The cloned laccase gene had identical sequences with 85% similarity as that of other whiterot fungi laccases and may be used for environmental pollutant degradation and wastewater treatment.

In conclusion the results from this study clearly show that laccase from the basidiomycete *Phanerochaete chrysosporium* (TL 1) is a new member of growing family of laccase enzymes possessing important properties for industrial applications.

REFERENCES

Bollag, D.M., M.D. Rozycki and S.J. Edelstein, 1996. Protein Methods. John Wiley and Sons Press, New York, USA.

Bourbonnais, R. and M.G. Paice, 1990. Oxidation of non-phenolic substrates: An expanded role for laccase in lignin biodegradation. FEBS Lett., 267: 99-102.

Calvo, A.M., G.C. Galletti and A.E. Gonzalez, 1995. Paper wastewater analysis by pyrolysis-gas chromatograph-mass spectrometry during biological decolorization with the fungi *Coriolopsis gallica* and *Paecilomyces vaiotii*. J. Applied Pyrolysis., 33: 39-50.

Das, N., Chakraborty and M. Mukherjee, 2001. Purification and characterization of a growth regulating laccase from *Pleurotus florida*. J. Basic Microbiol., 5: 261-267.

D'Souza, T.M., K. Boominathan and C. Adinarayana Reddy, 1996. Isolatoin of laccase gene specific sequences from white rot and brown rot fungi by PCR. Applied Environ. Microbiol., 62: 5307-5313.

D'Souza, T.M., C.S. Merritt and C.A. Reddy, 1999. Lignin modifying enzymes of the white-rot basidiomycete *Ganoderma lucidum*. Applied Environ. Microbiol., 65: 5307-5313.

Gawel, N.J. and R.L. Jarret, 1991. A modified CTAB DNA extraction procedure for musa and ipomea plant. Mol. Biol. Rep., 9: 262-266.

Herpoel, I., S. Moukha., L. Lesage-Meessen, J.C. Sigoillot and M. Asther 2000. Selection of *Pycnoporus cinnabarinus* strain for laccase production. FEMS Microbiol. Lett., 183: 301-306.

Higuchi, T., 1990. Lignin biochemistry: Biosynthesis and biodegradation. Wood Sci. Technol., 24: 23-63.

Kirk, T.K., W.J. Connors, R.D. Bleam, W.F. Hackett and J.G. Zeikus, 1975. Prepartion of microbial decompostion of ^{14}C synthetic lignin. Proceedings of National Academic Science, USA., 72: 2515-2519.

Marchuk, D., M. Drumm, A. Saulino and F.S. Collins, 1991. Construction of T vectors, a rapid and general system for direct cloning of unmodified PCR products. Nuclic Acids Res., 19: 1154-1156.

Martinez, D., Luis F. Larrondo, N. Putnam, D. Maarten, G. Sollewijn and J. Katherine, 2004. Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nature Biotechnol., 22: 695-700.

Min, L.L., Y.H. Kim., W. Kim., H.S. Jung and Y.C. Shah, 2001. Characterization of a novel laccase produced by the wood-rotting fungus, *Phellinus ribis*. Arch. Biochem. Biophys., 392: 279-286.

Pickard, M.A., R. Roman, R. Tinoco and R. Vazquez-Duhalt, 1999. Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. Applied Environ. Microbiol., 65: 3805-3809.

Ponting, S.B., E.B.G. Jones and L.L.P. Vrijmoed, 2000. Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture. Mycologia, 92: 139-144.

Saparrat, M.C.N., F. Guillen., A.M. Arambarri., A.T. Martinez and M.J. Martinez, 2002. Induction, isolation and characterisation of two Laccases from the white rot basidiomycete *Coriolopsis rigida*. Applied Environ. Microbiol., 68: 1534-1540.

Sealey, J. and A.J. Ragauskas, 1998. Residual lignin studies of laccase delignified kraft pulps. Enz. Microbiol. Technol., 23: 422-426.

Srinivasan, C., T.M. D'Souza., K. Boominathan and C.A. Reddy, 1995. Demonstration of laccase in the white rot basidiomycete *Phanerochaete chrysosporium* BKM-F1767. Applied Environ. Microbiol., 61: 4274-4277.

Xiao, Z., X.M. Tu, J. Wang, M. Zhang, Q. Cheng, K.Y. Zeng and Y.Y. Shi, 2003. Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete *Trametes* sp. strain AH28-2. Applied Microbiol. Biotechnol., 66: 700-707.

Yoropolov, A.I., O.V. Skorobogat'ko., S.S. Vartanov and S.D. Varfolomeyev, 1994. Laccase: properties, catalytic mechanism, and application. Applied Biochem. Biotechnol., 49: 257-280.