International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 10 (2014) pp. 213-227**
http://www.ijcmas.com

Original Research Article

Characterization and purification of laccase enzyme from *Aspergillus nidulans* CASVK3 from vellar estuary south east coast of India

K.E.Vivekanandan*, S. Sivaraj and S. Kumaresan

Centre for Advanced Study in Marine Biology Annamalai University,
Parangipettai 608502 Tamil nadu, India
**Corresponding author*

A B S T R A C T

The effects of carbon and nitrogen sources, initial pH and incubation temperature on laccase production by *Aspergillus nidulans* (KF974331) were evaluated using the one-factor-at-a-time method. The optimization of the *A. nidulans* produced 367.68U/ml using the Central composite design, and the molecular weight was obtained at 66kDa using SDS- PAGE. The analysis of purified laccase revealed that the optimized laccase produced showed a yield of 2.95 fold when purified by DEAE cellulose and the SDS-PAGE showed a band with a molecular weight of 66kDa and the ammonium sulfate precipitation showed a specific activity of 4.26U/mg protein and the yield was 62.9% with a purification factor of 1.35 fold.

Keywords

Aspergillus nidulans, Central composite design, SDS, ammonium sulphate precipitation

Introduction

Laccases (EC1.10.3.2), also named p-diphenol: dioxide oxidoreductases are blue multicopper oxidases (MCOs) that have the ability to catalyze the oxidation of a wide variety of organic aromatic compounds, concomitantly with the reduction of molecular oxygen to water (Ruiz-Duenas and Martinez 2009; Sakurai and Kataoka, 2007). The distribution of laccase enzyme is widespread among plants (Baldrain 2006), fungi (Abadulla *et al*., 2000a) and bacteria (Telke *et al*., 2010) which are involved in the physiological functions. In plants, they seem to be involved in lignin degradation, pigmentation and pathogenesis (Meyer and Staples 2002). Extensive studies made on fungal laccases have proven their potentiality (Arora and Sharma 2010), that could be tapped for paper-pulp bleaching (Aracri *et al*., 2011) synthetic dye decolorization (Murugesan *et al*., 2009), bioremediation, biosensor (Timur *et al*., 2004) and Immunoassays (Jordaan and Leukes 2003).

Redox mediators such as ABTS have been reported to enhance the degradation of many xenobiotics as well as increase laccase substrate range (Reyes *et al*., 1999; Call and Mucke, 1997). Laccase production is a common feature of many basidomycetes,

particularly those associated with wood decay or terminal stages of decomposition (Wood, 1980). Fungal laccases participate in conidial pigmentation and in infection processes during phytopathogenesis, to overcome the reactions of the host organism by polymerizing endogenous plant phenols, thus rendering them non-toxic (Mayer, 1987). However, the major role attributed to laccases in fungal species lies in the degradation of lignin and in the humification process (Hermann *et al*., 1983). In fungi, laccases are secreted and found intracellularly localised in the hulle cells and *Cleistothecial primordia* (Meideieros *et al*., 1999).

These enzymes can be produced by a variety of substances in various fungi independent of enzyme regulation, and the effects of inducers of laccase differ from fungus to fungus. Fungal laccases are involved in various processes in nature including the biodegradation of lignin (Eggert *et al*., 1996).The main objective of the present work was to study the optimization by one factor at a time approach and to study different purification and characterization techniques and their yield of specific activity and different sources like carbon, nitrogen. The SDS PAGE was used to find out the molecular weight of the enzyme purified and DEAE cellulose column chromatography was used to study the anions released by the *A.nidulans*.

Materials and Methods

Microorganism and inoculum preparation

The fungal strain was isolated in soil collected from mangrove region of the Vellar estuary. The geographical location of the study was carried out in Latitude of 11°029'134"N and Longitude of 79° 045'703"E of south east coast of India. The sediment was collected by using a sterile spatula and was put in Zip- lock bags and transported to the laboratory and immediately processed. The strain was grown in Potato dextrose Agar (PDA) slant, sub cultured periodically and stored at 4°C. The inoculum was prepared by fungal cultivation on a rotary shaker at 150 rpm in 250 ml flasks containing 100ml basal medium (g/l): Sucrose -10; Peptone – 5; KH_2Po_4- 1.0 ; NH_4NO_3-1.0; $MgSO_4$ -1.0 and Guaiocol-3mM and the following microelements were added to the basal medium (g/l) $FeCl_3$-1.0,$CoCl_2$-1.0, $ZnCl_2$-1.0, $CuSO_4.7H_2O$- 1.0; KCl- 1.0; $BaCl_2$-1.0; $HgCl_2$-1.0; $ZnSO_4$-1.0. Among the isolates strains, *Aspergillus nidulans* CASVK3 was selected for the production of laccase.

After 7 days of fungal cultivation, mycelial pellets were harvested and homogenized with a waring laboratory blender, three times for 20s with 1-min intervals (Mikiashvili *et al*., 2006). Each flask was inoculated using agar piece ($1cm^{-2}$) cut from an actively grown fungal culture. The flasks were incubated at 30°C on a rotary shaker (150 rpm). The culture broth was filtered, clarified by centrifugation at 10,000 rpm for 15 min, frozen, defrosted and then filtered to remove the precipitated polysaccharides. The resulting clear filtrate was used for the determination of soluble protein, enzyme activities, residual activity, and inducers effect on the laccase enzyme.

Determination of biomass

The mycelial biomass was harvested from the culture, it was washed with distilled water and pre weighed in a sterilized beaker. Then the biomass was dried at 105°C for 2 h and it was weighed in gram (Haq and Daud, 1995).

Laccase purification

The method for the laccase purification was adopted from the protocol described by Das *et al*. (2001) with minor modifications. All experiments were performed at 4° C unless and otherwise mentioned. The purification paramters calculations were carried out according to Nelson and Cox (2004)

Determination of molecular mass

Sodium dodecyl suphate polyacryalmide gel electophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). The same was used for the monitor the development of the purification process, to detrmine the homogenecity and apparent molecular mass of the purified laccase. SDS-PAGE was carried out on a 4% w/v stacking gel and 10% w/v seperating gel. The approximate molecular mass of the laccase was determined by calibration against broad range molecular weight markers, which contained the proteins 66, 45, 35, 20 kDa. The lacacse activity was exaimed with Guaiacol 1M as the substrate at different pH levels (6-12) by using appropriate buffers.

Ultrafiltration and Ammonium sulphate precipitation

The method for the ultrafiltration of laccase was adapted from a protocol described by Kim *et al*. (2002). The extracted culture filtrate was concentrated by ultrafiltration cell using Amicon 8200,YM-30 membrane through a membrane filter (molecular weight cut off 10kda) until a 10 fold concentration was achived. Concentrated filtrate was brought to 40% (w/v) saturation ($(NH_4)_2SO_4$ overnight at 4°C) then centrifuged at 6000xg for 30 min,the obtained precipitated pellet was discarded. The resulting supernatant was brought to 80% (w/v) saturation ($(NH_4)_2SO_4$, overnight at 4°C. The precipitate was collected and then resuspended in 100mM sodium phosphate buffer pH 6.0 and dialized against the same buffer overnight at 4°C. the dialyzed enzyme sample was subjected to anion exchange ch
romatography.

Anion exchange coloumn chromatography and Size –exclusion chromatography

The dialysate was loaded onto anion exchage (DEAE- cellulose) column (22x220mm) that had been pre-equilibrated with 100mM sodium phosphate buffer pH 6.0. The enzyme loaded column was washed with 500 ml of the same buffer to remove unbound sample components. A step wise gradient system of NaCl (0.2 to 1.0M) in the 100mM sodium acetate buffer (pH 6.0), was used to elute the bound protein at a rate of 1ml/min; fraction were collected and assayed for laccase activity. The active fractions of the laccase peaks were pooled together and dialyzed against the same buffer. The dialysate was subjected to size exclusion chromatography in the column (16x650mm) packed with Biogel P-200, are pre –equilibrated with 100mM sodium phosphate buffer pH 6.0. Active fraction were collected, assayed, pooled together and dialyzed against same buffer. The dialysate was concentrated by lyophilizer (Mini Lyodel Freeze Dryer, India) and stored at -20°C for futher characterization studies.

Effect of pH and temperature on laccase activity

The effect of pH was determined by measuring the activity at pH 3.0 to 6.0 in sodium acetate buffer, (100mM). The effect of temperature was determined by performing enzyme assays at temperature ranging from 20 to 80°C (sodium acetate

buffer, 100 mM, pH 5.0). For the determination of pH stability, the enzyme solution was diluted tenfold with 100mM sodium acetate buffer of pH values 3.0 to 6.0 and then incubated at 4°C. similarly, temperature stability was determined by incubating the enzyme solution in screw capped glass tubes at 30-70°C for different periods. The tubes were withdrawn at indicated times and cooled in ice water. In both cases the residual activities were determined at pH 5 and at 50°C.

Characterization of purified laccase
Effect of pH and Temeprature

The optimum pH of the purifed laccase enzyme was studied by incubating the laccase over a pH range of 3.5 to 10.0. The buffer system used were 100mM sodium acetate buffer for pH 3.5 to 5.5; 100mM sodium phosphate buffer pH 6.0-8.0; 100mM glycine-NaOH buffer pH 8.5-10.0. The purified laccase was incubated at the above pH for 30 min and the residual activity was determined spectrophotometrically at 470nm by guaiacol as the substrate. The temperature profile of the purified laccase was identified by incubating the enzyme for 30 min at different temperatures from 20 to 80°C with the increment of 10°C at the optimum pH determined by incubating the enzyme at temperature from 50 to 70°C with the increment of 5°C for different time period (1-5h). Residual activity was determined spectrophotometrically at 470nm using guaiacol as the substrate.

Effect of NaCl on enzyme stability and activity

Effect of NaCl on purified laccase was determined at different concentrations of NaCl ranging from 0.0 to 2.0M. Salt tolerance of the purified laccase was determined by incubating at different concentrations of NaCl, upto 2M for 24h at 45°C, the residual activity was calculated under the satandard assay conditions at frequent time intervals. Laccase activity in the absence of NaCl was also determined fo r comparison.

Effect of substrate on purified laccase activity

Aromatic substrates namely ABTS, guaiacol, pyrogallol, *p*-phenylenediamine, catechol, ferulic acid, tyrosine, and veratryl alcohol were chosen to study laccase substrate specificity (Murugesan et al., 2006). These compounds were tested at 1 mM concentrations separately with sodium acetate buffer (100 mM, pH 6.0) using 10 µg of laccase enzyme. The enzyme assays were done as described earlier.

Effect of metal ions on purified laccase activity

The effect of metal ions on the purified laccase was determined to check the stability and the relative activty of the metals like Copper, Calcium, Potassium, Magnesium, Ferric ion, Zinc and some heavy metals like Cobalt, Nickel and Mercury and Lead (each at 1mM to2mM) concentration.

Effect of different inhibitors on activity of purified laccase

The effects of several potential inhibitors were determined by incubating the purified laccase with various concentrations of inhibitors and measured the residual activity with guaiacol as substrate. L-cysteine,sodium metabisulphite, sodium sulphite, sodium dithionite was incubated with the purified laccase at four different concentrations (0.1,2,5,10mM) for 30 min at

room temperture. The change in absorbance was measured spectrophotometrically at 470nm. A control test was conducted in parallel in the absence of the inhibitor.

Kinetic constants against the purified laccase enzyme

Kinetic constants of laccase for the most commonly used substrates are guaiacol, ABTS, 1-napthol, were investigated. The reactions were conducted at standard assay condition. The wavelengths of laccase activity with the above mentioned substrates were determined spectrophotometrically by allowing the reactions of the substrates with laccase to proceed to completion, performing a spectral scan and using suitable λmax (wavelength of maximum absorbtion). Kinetic studies were conducted for the selected four substrates and the Vmax and Km values were calculated using the Michaelis Menten equation.

Results and Discussion

Among the isolates, *Aspergillus nidulans* CASVK3 showed relatively higher laccase activity than that of other isolates (Fig-1) and the strain identified by 16S rDNA sequence and obtained accession number KF974331. Therefore, *Aspergillus nidulans* CASVK3 was selected for further experiment. The strain produced 367.68U/ml of laccase enzyme when optimized using different carbon and nitrogen sources (Fig-2 and Fig-3).

Biomass determination

The growth of the fungus *Aspergillus nidulans* and the production of enzyme were highly influenced by effective controlling factors like pH, Temperature and NaCl concentration and the laccase activity and biomass was studied at different time (Days) and pH, NaCl (Fig-4.a and Fig- 4.b and Fig-4.c, 4d). The growth of organism in the production medium without any specific parameters and it was considered as control.

Purification and characterization of laccase enzyme

In order to identify the laccase enzyme produced by *Aspergillus nidulans*, crude laccase was subjected to SDS PAGE. *A. nidulans* producing laccase enzyme of molecular weight of 66kDa (Fig-5). The (Table.1) shows the purification summary of the ammonium sulfate precipitation, the specifc activity was increased to 4.26U/mg protein and the yeild was 62.9% with a purification factor of 1.35 fold. The dialyzed sample was applied to a DEAE-cellulose column chromatography, the specific activity was increased to 7.16U/mg protein and the yeild was 50.5% with purification factor of 2.95 fold. Fraction with laccase activity were pooled and dialyzed without any apparent loss of activity and loaded onto a Biogel P-200 column. At the end of the purification process, laccase enzyme was purified to 9.19 U/mg of protein using guaiacol as substrate under standard assay condition. The purified enzyme yeilded a single band in SDS-PAGE after staining with Coomassie brilliant blue R-250 and guaiacol, respectively molecular weight of the laccase enzyme was calculated to be ~66kDa.

Effects of pH and temperature on the activity of Aspergillus nidulans

The influence of pH within the range of 3.0 to 11 on laccase activity of *A. nidulans* was studied and the results were plotted. The effect of temperature in the production of laccase and biomass in the shake flask was shown in (Table.2).

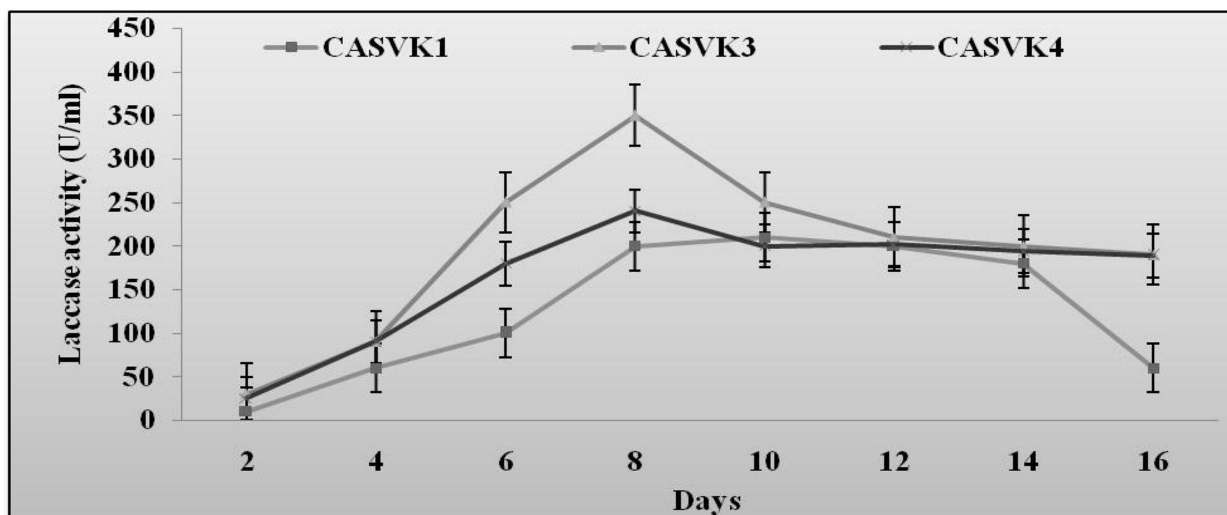

Figure.1 Oxidative polymerization of potential strains in Czapek dox broth

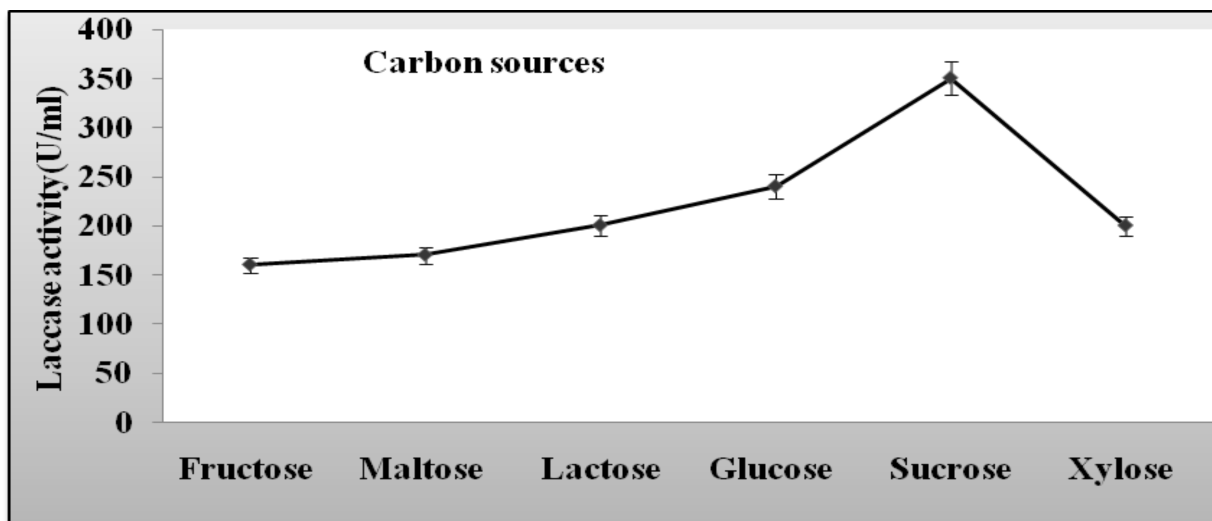

Figure.2 Effect of different carbon sources on Laccase production by *A. nidulans*

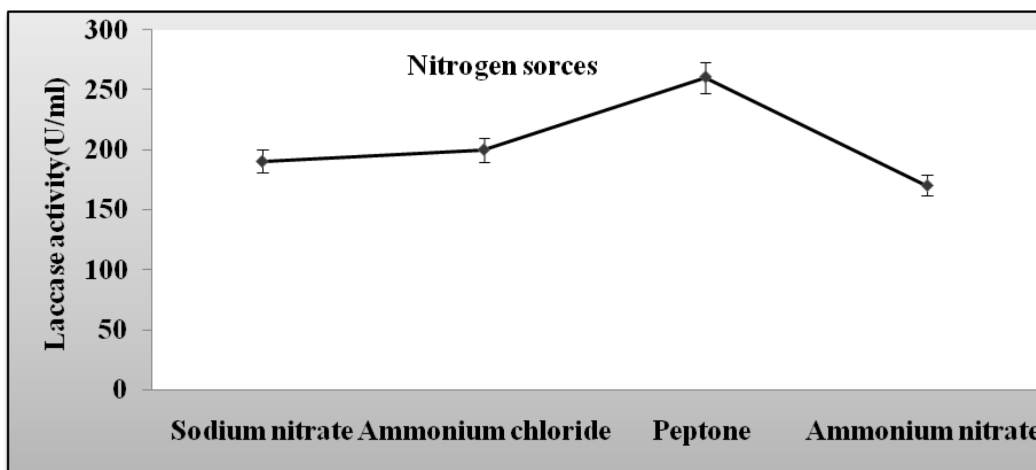

Figure.3 Effect of different Nitrogen sources on Laccase production by *A. nidulans*

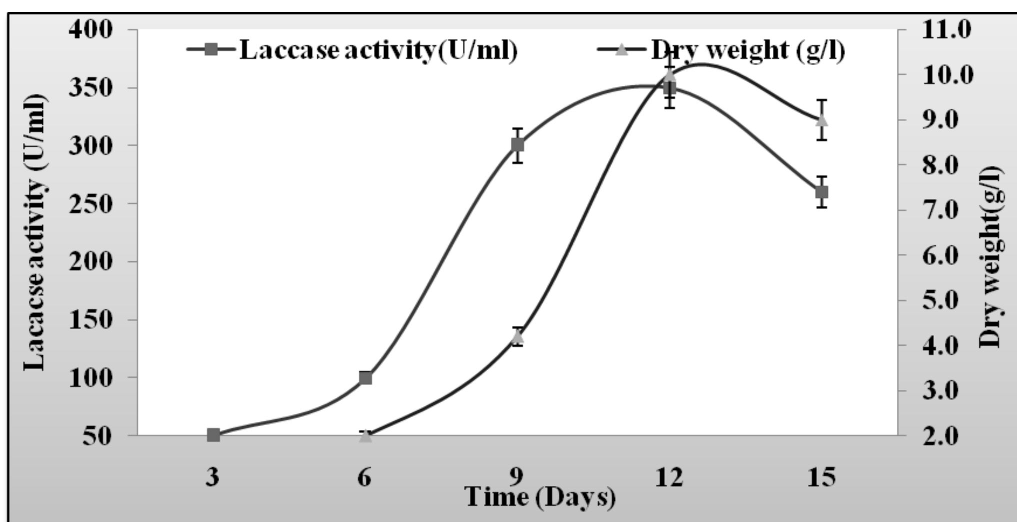

Figure 4.a Relative activity of laccase enzyme against the biomass in different days interval

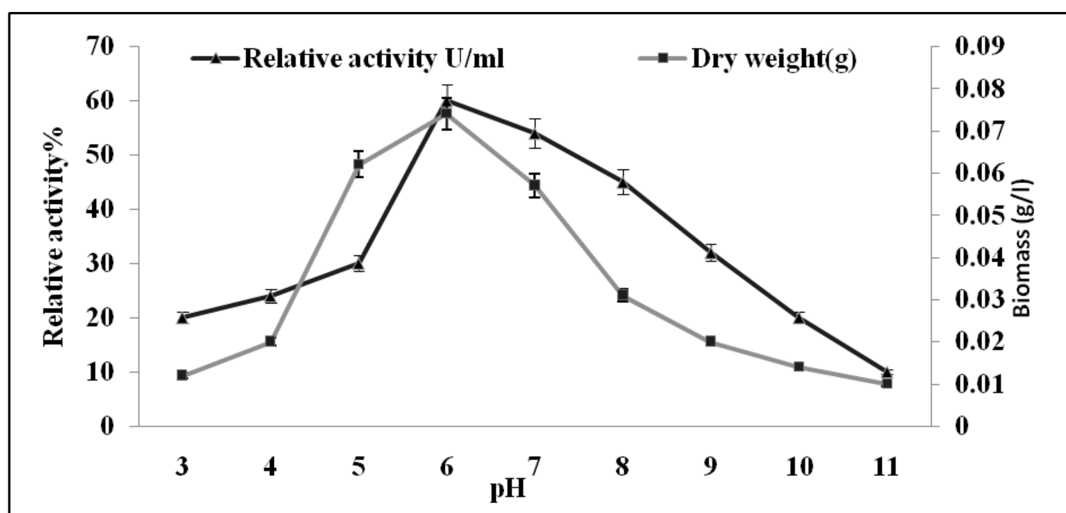

Figure 4.b Relative activity of laccase enzyme against the biomass in different pH

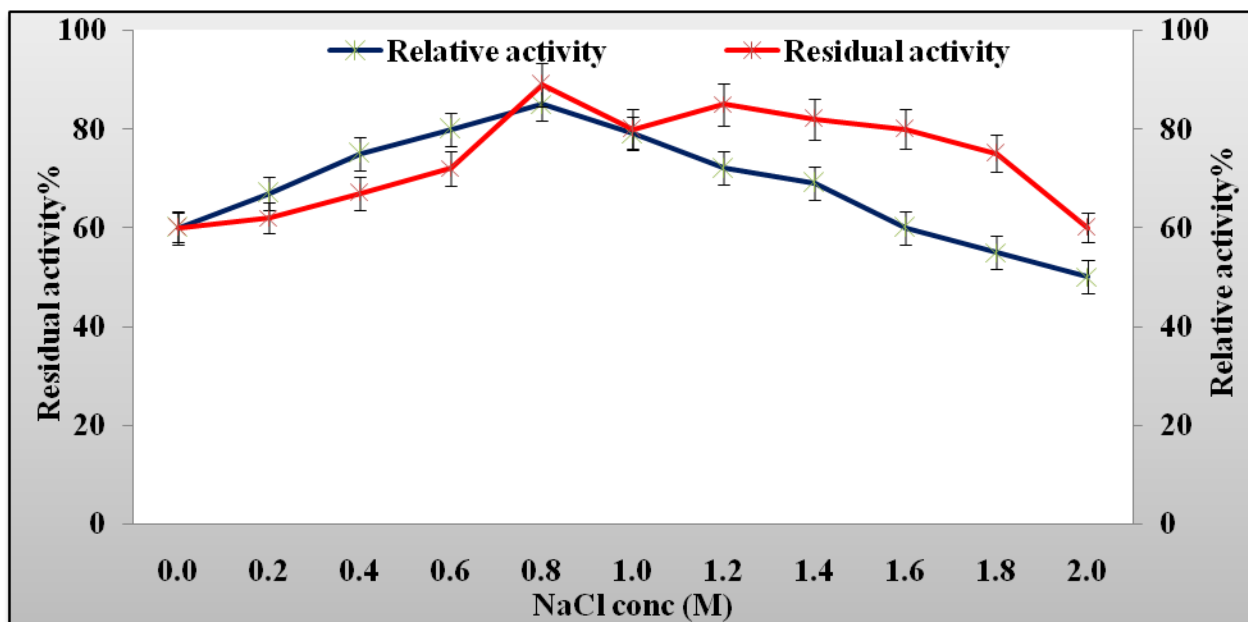

Figure 4.c Effect of NaCl on purified laccase of *A. nidulans*

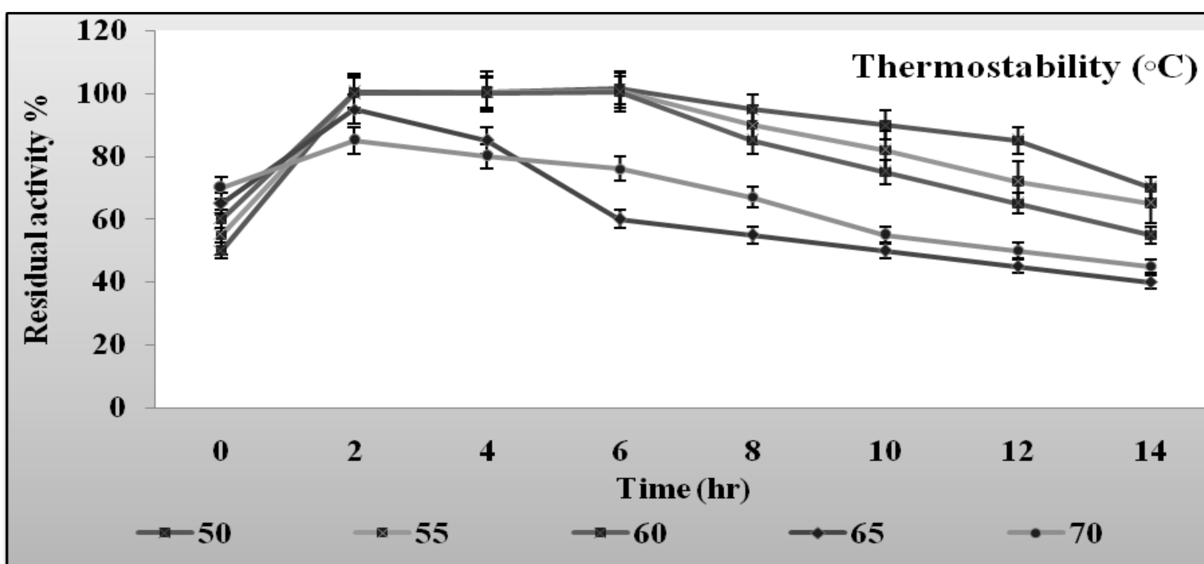

Figure 4.d Thermostability of the laccase produced by *A. nidulans* at different time duration

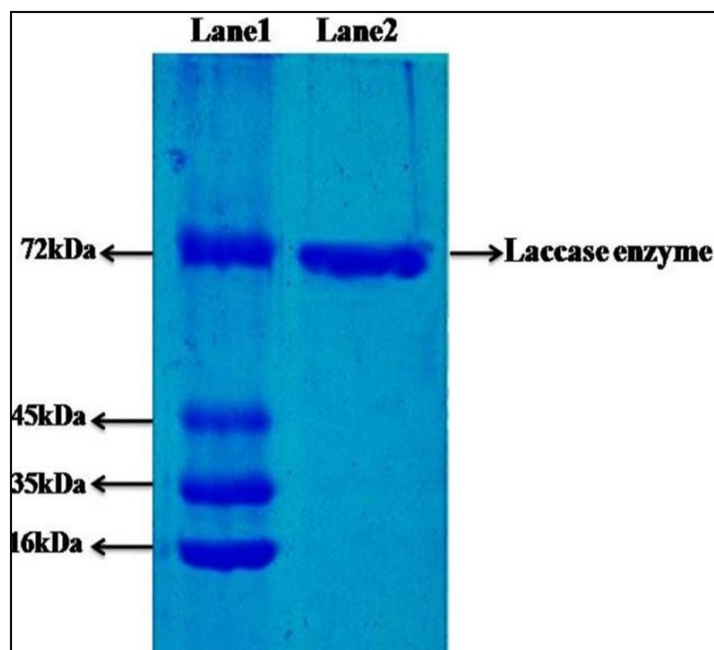

Figure.5 Molecular weight determination of purified laccase enzyme (66 kDa) on SDS-PAGE analysis

Table.1 Summary and purification procedure of *A. nidulans* laccase enzyme

Purification steps	Total laccase (U/ml)	Total Protein (mg)	Specific activity of laccase (U/mg)	Yeild	Purification factor (fold)
Crude enzyme (culture filtrate)	23702	75	340	100	1
Ultrafiltration (5kDa cut off)	129712	12	11125	545	34
Ammonium sulfate salt precipitation (80%)	560	150	4.56	62.9	1.35
DEAE –Cellulose	430	60	7.16	50.5	2.95
Biogel P-200 Laccase enzyme	330	30	9.19	42.45	5.8

Table.2 The influence of temperature on the laccase and biomass production from *A.*nidulans

S. No	Temperature ◦C	pH	Biomass (g/l)	Laccase (U/ml)	Q^a_p (U/ml/h)
1	20	6.0	20.5±3.0	224±4.9	3.52
2	25	6.0	18.2±3.5	350±6.1	2.36
3	30	6.0	17.5±2.7	200±4.2	2.22
4	35	6.0	13.6±1.2	150±4.0	1.49

Table.3 The influence of pH on the laccase and biomass production by *A. nidulans*

S. No	Temperature ◦C	pH	Biomass (g/l)	Laccase (U/ml)	Q^a_p (U/ml/h)
1	30	5.0	20.5±3.0	220±4.9	3.52
2	30	5.5	18.2±3.5	350±6.1	2.36
3	30	6.0	17.5±2.7	200±4.2	2.22
4	30	6.5	13.6±1.2	180±4.0	1.49

Table 4. Effect of metal ions on purified laccase from *A. nidulans*

Metal ions	Concentration(mM)	Relative activity(%)
Control	-	100
Cu	2.0	168
Ca	2.0	95
K	2.0	106
Mg	2.0	119
Fe	2.0	139
Zn	2.0	79
Co	1.0	25
Ni	1.0	32
Hg	1.0	19
Pb	1.0	20

Table.5 Kinetic parameters for *A. nidulans* laccase enzyme

Substrate	Wavelength	pH	K_m(mM)	V_{max}(mM/Sec)	V_{max}/K_m
Guaiacol	470	6.0	0.052	2.86	55
ABTS	436	5.5	0.072	1.53	18.4
1- Napthol	425	5.0	0.125	0.94	7.52

Note: The enzyme activity was performed at 60°C. All the values were calculated by the linear regression (correaltion coefficent ≥0.98) of double reciprocal plots, $1/v_0$ versus 1/[s], from every set of triplicate measurements.

Table.6 Effect of inhibitors on *A. nidulans* laccase enzyme

Inhibitor	Concentration (mM)	Inhibition (%)
L-cystine	0.1	98
	2	99
	5	100
	10	100
Sodium sulphite	0.1	2
	2	16
	5	70
	10	100
Sodium hydrogen sulphite	0.1	34
	2	70
	5	100
	10	100
Sodium metabisulphite	0.1	80
	2	99
	5	100
	10	100
Sodium dithionite	0.1	65
	2	98
	5	100
	10	100

Experimental data suggest that the increased production of laccase was obtained at 25˚C when the initial pH was 6.0. The maximum production of laccase 367.67 U/ml was obtained when the pH of the medium was not stabilized. pH is one of the important factor, which plays a major role in the production of laccase. Three levels of pH were studied by maintaining the temperature constantly at 30˚C. The maximum production of laccase and biomass were attained at pH 6.0, the obtained laccase activity and biomass are 367.67U/ml and18.2±3.5 g/l respectively. The optimum pH for laccase activity was found to be 5.0 (1.19 U/ml). The activity of laccase was decreased with increasing pH after 5.

The volumetric production rate (Q_p^a) of laccase was determined as 3.52 U/ml/hr .When the temperature was maintained at 30˚C could be compensated by the lower energy and cost required for temperature control. The optimum temperature for lacasse activity was found to be 40°C (1.09 U/mg). Further, the increase of temperature above 40°C reduces the enzyme activity considerably.

The optimum pH was not identical to the other substrates like ABTS, 1-napthol and pyrocatechol. The laccase shows optimum pH 5.5 for the substrates 1-napthol and pyrocatechol. The optimum pH for the pyrocatechol was 6.0. When pH values greater than 6.0, the enzyme activity decreased gradually and completley inactivated at higher alkaline pH. The residual activity of purified laccase enzyme of *A.nidulans* was determined at various temperatures (20 to 60˚C) at pH 5.5 using guaiacol as substrate. Maximum activity was

observed at 45°C. Considerable activity was retained also at 50°C and at higher temepratures of 55 and 60°C, the residual activities were reduced to 85% and 69% respectively. Thermo stability studies showed that purified laccase was not stable at temperatures higher than 50°C over the prolonged incubation.

The result of halotolerance studies on the purified lacacse shows that, the enzyme was active at different concentrations of NaCl within the tested range. Activity at low and high concentrations of NaCl indicated that the purified lacacse was a halo-tolerant enzyme. Maximum activity was obtained at 0.8 M NaCl concentration, the enzyme exhibited 40% additional activity in the absence of NaCl (control). The enzyme was found to be stable at NaCl concentrations upto 1.2M, the stability was gradually decreasing at concentrations higher than 1.2M NaCl even though 66% residual activity was retained at 2M NaCl (Fig-4.c) concentration. It was obvious from the study that there was no change in the stability of enzyme over different incubation time. The activities retained after 1h,12h,and 24h (residual activities) were almost the same, which implied that the reaction of enzyme with salt was relatively rapid and no futher loss of activity was possible over long incubation time. The effects of various metal ions on the activity of purified laccase is given in (Table 3). The enzyme activity was enhanced by metal ions such as Fe,Cu,Ca,Zn,Mg,K,Na,Mn (each at 2mM) while the heavy metals like Hg, Cd,Co,Pb,Ni reduced the activity considerably, even at half the concentration (1.0mM) of other metal ions. The most obvious effect on laccase activty was exerted by Copper,which enhanced the activity at the same level.

As mentioned in the methods, the purified laccase was characterized in terms of its affinity constant (K_m) and maximum velocity constant (V_{max}) of the three different substrates namely guaiacol, and ABTS (Table .4). The enzyme showed greater affinity towards guaiacol (0.052mM) and ABTS (0.072mM) respectively. Reactions were initiated by addition of laccase and initial rates were obtained from the linear portion of the progress curve. The fraction without enzyme served as the control. Effect of range of potent laccase inhibitors on the laccase activity was tested with guaiacol as substrates and the results are presented in (Table. 5) and inhibitors were added to the assay mixture at different concentrataions. After incubation (30 min) substrate was added and the residual enzyme activity was determined. Enzyme inhibition was expressed in percentage. The enzyme shows affinity towards guaiacol (0.052mM) when compared with K_m value of guaiacol (2.86mM). The inhibitors like L-cystine, sodium dithionite, sodium sulphite needs 10mM concentration to completely inhibit the enzyme activity

In the present study many isolates were identified and *Aspergillus nidulans* was found to be effective in laccase production when screened. Similarly, Rajeshwari and Paravatham (2011) found that *Aspergillus* sp. produced (9.21U/ml) on 10th day of the incubation.

The lower pH condition can affect fungal growth to reduce such response and further inhibit the metabolism of laccase synthesis (Galhaup *et al*., 2002). In addition, the condition with a low pH level can also promote the changes of the three-dimensioned structure of the enzyme (Tavares *et al*., 2006) Whereas, Nyanhongo et al., (2002) observed that the increase in biomass of *Trametes modesta* was proportional to the increase in laccase

activity and glucose was almost completely utilized after the fourth day of incubation. Murugesan *et al*., (2006) reported that optimum temperature of laccase from *Pleurotus sajor-caju* was 40°C. However, other fungal laccase have optimum temperature ranging from 20°C in *Ganoderma lucidum* (Ko et al., 2001) and 80°C in *P. eryngii* (Choi *et al*., 1995). However 44 percent activity was retained at pH 8.

The purified laccase showed optimum activity at pH 5.0 is in concordance to the optimum pH observed for *L. lividus* laccase (Sahay *et al*., 2009). The laccase from Ascomycetes, *Aspergillus* sp. showed optimal activity at pH 5.0 and most stable at pH 8 which was in par with Xu et al. (1996) who stated that laccase from Basidomycetes had optimal pH of activity in acidic pH and stability at neutral or alkaline pH. The molecular masses of purified laccase was not consistent with most of the *Aspergillus* species namely the *A.oryzae* on SDS-PAGE the induced wt-lcc4 appears to have a MWt (molecular weight) of about 66kDa while r-lcc4 migrates as a smear over 70-85kDa (Jill *et al*.,1996) and *R. practicola* had a molecular weight of about 78kDa (Bollag *et al*.,1979). The laccases from basidomycetes including Trametes species are generally monomeric proteins with molecular mass between 50 and 80kDa (Levin *et al*.,2002 and Yaropolov *et al*., 1994). The K_m value for laccases from the fungi usually ranges from 0.01 to 0.6mM (Galhaup *et al*.,2002; Saito *et al*., 2003).

Lu *et al* (2007a) and Baldrain (2004) reported that L-cystine was one of the effective inhitor of fungal laccase. However, Johannes and Majcherczyk (2000) showed that the observed inhibitory effect was actually caused by the reduction of the oxidized substrate by the sulfhydryl compounds and not by true inhibition of the enzyme. Laccases can be inhibited when the inhibitor vinds to the strongly and stopping futher catalysis of the reaction. This occurs when the Cu at the catalytic center is removed/chelated or by competting for O_2, which is specific co substrate of laccase. Laccases have been known to be inhibited by diethyl dithiocarbamate and thiogycolic acid probably due to their effect on copper at the catalytic centre of laccase and by several sulfhydryl compounds such as dithiothreitol, thiogycolic acid, cystine and ditethyldithiocarbamic acid (Baldrain, 2006).

The present study proposes that the non white rot fungi is capable of producing Laccase enzyme which is a powerful lignocellulosic enzyme, used in many industries for bioleaching and dye degrading industries. In the present study, RSM and Plackett Burman design was used to establish an optimized culture condition for laccase production and the sole carbon source was sucrose and the nitrogen source was peptone, which yeild the purified laccase enzyme (367.68U/mL) and many purification steps was employed to get a better fold of increase in the yeild of production. The thermostability and the effect of salinity proved to be more tolerent thus proving as halotolerent enzyme.

Acknowledgement

Authors are thankful to Dr. K. Kathiresan, Dean and Director CAS in Marine Biology, Annamalai University and Prof. T. Balasubramanian, former Director and Dean, and also thankful to Ministry of Earth Sciences (MoES-SIBER) Sustainable Biogeochemistry Ecosystem Research for funding support.

References

Abadulla, E., Tzanov, T., Costa, S., Robra, K.H., Paulo, A.C., Gubitz, G.M., 2000a.
Decolorization and detoxification of textile dyes with a laccase from Trametes hirsuta. Appl. Environ. Microb. 66, 3357 – 3362.

Aracri, E., Colom, J.F., Vidal, T., 2009. Application of laccase-natural mediator systems to sisal pulp: an effective approach to biobleaching or functionalizing pulp fibres? Bioresour. Technol. 100, 5911 – 5916.

Arora, D.S., Sharma, R.K., 2010. Ligninolytic fungal laccases and their biotechnological applications. Appl. Biochem. Biotech. 160, 1760 – 1788.

Baldrian, P., 2004. Purification and characterization of laccase from the white-rot fungus Daedalea quercina and decolorization of synthetic dyes by the enzyme. J. Appl. Microbiol. Biotech. 63, 560 – 563.

Baldrian, P., 2006. Fungal laccases – occurrence and properties. FEMS. Microbiol. Rev. 30, 215 – 242.

BolIag, J.-M., R. D. Sjoblad, and S.-Y. Liu. 1979. Characterization of an enzyme from *Rhizoctonia praticola* which polymerizes phenolic compounds. Can. J. Microbiol. 25:229-233.

Choi, Y .O., Kim, S.J. and Choi, H.T., 1995. Purification and characterization of laccase from Coprinus congregates. Microorg. Ind. 21: 351-358.

Das, N., Chakraborty, T.K., Mukherjee, M., 2001. Purification and characterization of a growth regulating laccase from Pleurotus florida. J. Basic Microbiol. 41, 261 - 267.

Eggert, C., Temp, U., Eriksson, K.-E.L., 1996. The ligninolytic system of the white rot fungus Pycnoporus cinnabarinus: purification and characterization of the laccase. Appl. Environ. Microbiol.62:1151 – 8.

Francisco J. Ruiz-Dueñas and Angel T. Martínez (2009)Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this Microbial Biotechnology 2(2), 164–177

Galhaup, C., Goller, S., Peterbauer, C.K., Strauss, J., Haltrich, D., 2002. Characterization of the major laccase isoenzyme from Trametes pubescensand regulation of its synthesi by metal ions. Microbiology 148, 2159-2169.

Haq, P.B., Daud, D.A., 1995. Process of mycelial dry weight calculation for citric acid. J. Biotechnol. 9, 31 – 35

Hermann, T.E., Kurtz, M.B., Champe, S.P., 1983. Laccase localised in hulle cells and cleistothecial primordia of *Aspergillus nidulans*. J. Bacteriol. 154, 955–964.

Johannes, C., Majcherczyk, A., H€ uttermann, A., 1996. Degradation of anthracene by laccase Of *Trametes versicolor* in the presence of different mediator compounds. Appl. Microbiol. Biotechnol. 46, 313-317.

Khuri, A.I., Cornell, J.A., 1987. Response Surfaces. Dekker, New York, NY.

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

Ko, E.M., Leem, Y .E. and Choi, H.T., 2001. Purification and characterization o of laccase from the white-rot basidiomycete *Ganoderma lucidum*. Appl. Microbiol. Biotechnol. 57: 98-102.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227: 680-685.

Levin L, Forchiassin F, Ramos AM. Copper induction of ligninmodifying enzymes in the white-rot fungusTrametes trogii. Mycologia 2002;94:377–83.

Lowry, O.H., Rosebrough, N.J and Randal, R.J., 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265-275.

Lu, L., Zhao, M., Zhang, B.B., Yu, S.Y., Bian, X.J., Wang, W., Wang, Y., 2007a. Purification and characterization of laccase from Pycnoporus sanguineus and decolorization of an anthraquinone dye by the enzyme. Appl. Microbiol. Biot. 74, 1232 -1239.

Mayer, A. M. 1987. Polyphenol oxidases in plants: recent progress. Phytochemistry 26:11-20.

Meideieros, M.B., Bento, A.V., Nunes, A.L.L., Oliveira, S.C., 1999. Optimization of some

variables that affect the synthesis of laccase by Pleuratus ostreaties. Biopol. Eng. 71, 483–487

Mayer, A.M., Staples, R.C., 2002. Laccase: new functions for an old enzyme. Phytochemistry 60, 551-565.

Mikiashvili, N.,Wasser, S.P., Nevo, E., Elisashvili, V., 2006. Effects of carbon and nitrogen sources on Pleurotus ostreatus ligninolytic enzyme activity. World J. Microbiol. Biotechnol. 22, 999-1002.

Murugesan, K., Kim, Y.M., Jeon, J.R., Chang, Y.S., 2009. Effect of metal ions on reactive dye decolorization by laccase from Ganoderma lucidum. J. Hazard. Mater. 168, 523 – 529.

Murugesan, K., Arulmani, M., Nam, I.H., Kim, Y.M., Chang, Y.S. and Kalichelvan, P.T., 2006. Purification and characterization of laccase produced by white rot fungi *Pleurotus sajor caju* under submerged culture condition and its potential in decolorization of azo dyes. Appl. Microbiol. Biotechnol. 72: 939-946.

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

Nyanhongo GS, Gomes J, Gübitz G, Zvauya R, Read JS, Steiner W. Production of laccase by a newly isolated strain of *Trametes modesta*. Bioresour Technol 2002;84:259–63.

Rajeswari, M. and Parvatham, R., 2011. Purification and Characterization of Laccase from *Aspergillus* Sp. Adv Bio T ech. :1 1(5): 28-31.

Sahay, R., Yadav, R.S., Yadav, K.D., 2009. Purification and characterization of laccase secreated by *L.lividus*. Appl. Biochem. Biotech. 157, 311 – 320.

Saito, T., Hong, P., Kato, K., Okazaki, M., Inagaki, H., Maeda, S., Yokogawa, Y., 2003.

Purification and characterization of an extracellular laccase of a fungus (family Chaetomiaceae) isolated from soil. Enzyme Microb. Tech. 33, 520 – 526.

Sakurai, T., Kataoka, K., 2007. Structure and function of type I copper in multicopper oxidases. Cell. Mol. Life Sci. 64, 2642-2656.

Smith, M., Thurston, C. F., Wood, D.A., 1997. Fungal laccases: role in delignification and possible industrial applications. In Multi-copper oxidases Edited by: Messerschmidt A., Singapore, World Scientific. 201-224.

Tavares, A.P.M., Coelho, M.A.Z., Agapito, M.S.M., Coutinho, J.A.P., Xavier, A.M.R.B., 2006.

Optimization and modeling of laccase production by *Trametes versicolor* in a bioreactor using statistical experimental design. Appl. Biochem. Biotechnol. 134, 233-248.

Telke, A.A., Joshi, S.M., Jadhav, S.U., Tamboli, D.P., Govindwar, S.P., 2010. Decolorization and detoxification of Congo Red and textile industry effluent by an isolated bacterium *Pseudomonas sp*. SU-EBT. Biodegradation. 21, 283 – 296.

Timur S, Pazarlıoglu N, Pilloton R, Telefoncu A. Thick film sensors based on laccases from different sources immobilized in polyani-line matrix. Sens Actuators B Chem 2004;97:132–136.

Wood, D.A., 1980. Production Purification and properties of extra- celluar laccase of Agaricus bisporous. J. Gen. Microbiol. 117, 327– 338.

Yi-Rui Wua, Ding-Lan Nian. Production optimization and molecular structure characterization of a newly isolated novel laccase from *Fusarium solani* MAS2, an anthracene-degrading fungus International Biodeterioration & Biodegradation 86 (2014) 382- 389.

Xu, F., Shin, W., Brown, S.H., Wahleitner, J.A., Sundaram, U.M., Solomon, E.I., 1996. A study of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity and stability. Biochim. Biophys. Acta. 1292, 303 – 311.

Yaropolov AI, Skorobogat'ko OV, Vartanov SS, Varfolomeyev SD. Laccase. Properties, catalytic mechanism, and applicability. Appl Biochem Biotechnol 1994;49:257–80.