Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 24, No. (3) : 2022 : 528-538

ISSN-0972-3005

DOI No.: http://doi.org/10.53550/AJMBES.2022.v24i03.0016

PURIFICATION AND CHARACTERIZATION OF LACCASE FROM ARTHROGRAPHIS *KSF*$_2$

SHEENA DEVASIA* AND A. JAYAKUMARAN NAIR

Department Of Biotechnology, University Of Kerala, Kariavattom, Trivandrum, Kerala, India

(Received 29 March, 2022; Accepted 20 May, 2022)

Key words: Oxidoreductase, Laccase, Arthrographis, ABTS, Yellow laccase

Abstract– Laccases are blue copper oxidases (E.C. 1.10.3.2 benzenediol: oxygen oxidoreductase) that catalyze the one-electron oxidation of phenolics, aromatic amines, and other electron-rich substrates with the concomitant reduction of O_2 to H_2O. They are currently seen as highly interesting industrial enzymes because of their broad substrate specificity. An ascomycetes strain, Arthrographis KSF_2 was isolated and enzyme was purified and characterized. Laccase activity was determined using ABTS as substrate. Laccase was purified by ion-exchange and gel filtration chromatography. The purified laccase showed a molecular weight of 55 kDa and pI of 5.5 when analyzed by 2-D PAGE. The purified enzyme was even stable at pH 11 and it was observed that there was 74.4% retention of activity at pH 11 after 24hrs. The K(m) and V(max) values are 15 (μM) and 21.9 (μmol/min), respectively, with ABTS as substrate. The effect of 5mM metal ions such as Mn^{2+}, Mg^{2+}, Na^{+}, Ca^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+} was estimated after incubating the enzyme with the metal ion for 5 min. It was observed that $FeCl_3$ inhibited the laccase activity to the maximum (70.5%) whereas the enzyme was least inhibited by $MnSO_4$ (21%). It was observed that even $CuSO_4$ inhibited the laccase activity in 5mM concentration. There was 100% denaturation by 1% SDS in 10 min whereas only 8% denaturation was observed by Tween 20. Tween 80 denatured 21% enzyme and Triton X 100 denatured 25% of the total enzyme. The purified laccase on UV-Vis analysis did not show the characteristic laccase peak at 600nm and so it was confirmed as a typical yellow laccase.

INTRODUCTION

Many laccase producing fungi secrete isoforms of the same enzyme (Leontievsky *et al.,* 1997). These isozymes have been found to originate from the same or different genes encoding for the laccase enzyme (Archibald *et al.,* 1997). The number of isozymes present differs between species and also within species depending on whether they are induced or non-induced (Assavanig *et al.,* 1992). They can differ markedly in their stability, optimal pH and temperature and affinity for different substrates (Assavanig *et al.,* 1992, Heinzkill *et al.,* 1998). Furthermore, these different isozymes can modulate different roles in the physiology of different species or in the same species under different conditions (Assavanig *et al.,* 1992). *Cerrena unicolor* secreted two laccase isoforms with different characteristics during the growth in a synthetic low-nutrient nitrogen/glucose medium (Michniewicz *et al.,* 2006). All enzyme producing facilities have been used the established approach of SmF owing to better monitoring and ease of handling (Singhania *et al.,* 2010). Disadvantages of SSF over SmF are difficulty in scale-up and control of process parameters (pH, heat, nutritional requirements, etc.), higher impurity product, increased cost in recovering product etc (Holker and Lenz, 2005; Robinson *et al.,* 2001; Sandhya *et al.,* 2005). Thereby, SSF will never replace the established processes for the production of enzymes and metabolites optimized in SmF conditions (Holker and Lenz, 2005). Ramamurthy and Kothari (1993) observed that surface cultivation led to protease of low specific activity when compared to SmF and production cost was also higher.

MATERIALS AND METHODS

Production of laccase enzyme

Submerged fermentation technique was selected as

the mode of enzyme production in purification studies. *Arthrographis* KSF_2 was inoculated to the optimized VMM (Devasia S and Nair A J, 2016a). 1000 ml of the modified VMM media was prepared and *Arthrographis* KSF_2 strain was inoculated into the production media. The strain was inoculated to the media from a 10 day old VMM agar plate. Enzyme samples were taken in every 48 hours and laccase assay was conducted using ABTS as substrate. The specific activity was noted and the purification was conducted on the 25th day of inoculation. The supernatant was harvested by filtration through sterile filter papers and the filtrate was subjected to fractional precipitation.

Enzyme assay and protein estimation of the pellet and supernatant samples were conducted using ABTS as substrate. The fractions were subjected to dialysis using 0.01 M pH 5 citrate buffer with 4 consecutive buffer changes. The dialyzed samples were lyophilised to a constant volume. Enzyme assay and protein estimation was conducted after each purification step.

Analysis of the isoenzyme pattern of Arthrographis KSF2 laccase by Native PAGE

The ammonium sulphate fractions (60%, 80%, 100%) was taken for native gel analysis (zymogram) and SDS PAGE. 45µg of protein was loaded into each well and the gel was run at a constant current of 25 mA. The native gel was stained using 0.1M acetate buffer (pH 4.5) containing 1mM guaiacol (Jia Li Dong *et al.,* 2004).

Ion Exchange Chromatography (IEC)

DEAE column, with a flow rate of 1-1.5 ml/min was used. The concentrated protein sample was used for loading the column. The volume of the sample used for sample loading was 1/100th of the matrix volume. Protein was eluted using increasing gradient of NaCl in the elution buffer (10mM Tris-HCl buffer pH 8.2 with 0.01M - 1M NaCl). Fractions (3ml each) were collected in sterile vials. A280 of the fractions was read and the peaks were noted and a chromatogram was prepared. ABTS assay of each of the peaks was done to find the gradient of NaCl were the protein of interest was eluted. The fractions containing maximum laccase activity and minimum protein concentration was pooled and concentrated by 10KD ultrafiltration membranes (Amicon, Millipore). The samples were centrifuged at 7500rpm for 10min at 4°C. The concentrated sample with laccase activity was then subjected Gel Permeation Chromatography (GPC).

Gel Permeation Chromatography (GPC)

The sample is concentrated to a small volume for effective resolution. The volume of matrix was 30 to 100 times the sample load volume. A constant flow rate of 1-1.5 ml/min was maintained throughout the column run. A280 of each sample was analyzed for the protein content. A chromatogram was prepared and the peaks were taken for ABTS assay. Column wash was continued using buffer with 1M salt until the absorbance at 280nm was zero. Fractions with maximum activity and minimum protein were pooled and Ultra Filtration was conducted.

Percentage of the yield of the enzyme after each purification step and the purification fold of laccase was calculated.

PAGE Analysis

The mw of purified laccase was estimated by 12% SDS PAGE under denaturing conditions. 45µg of denatured protein samples were loaded the gel was run at a constant current of 25mA. Silver Staining of the gel was performed. The gels were photographed in a gel documentation system and stored in the storage solution. Zymogram analysis of purified laccase was also conducted and guaiacol staining was performed.

Two Dimensional Polyacrylamide Gel Electophoresis

2D electrophoresis was done using the Bio-Rad Fast Gel System. The concentrated GPC fraction with maximum purity and activity was selected for IEF. The mw and iso electric point of laccase was determined by comparing with an IEF protein standard (Biorad). Appropriate volume of the sample (4-8µg protein) and rehydration solution mixture is mixed. 125 µl of the mixture is applied on the strip holder. The gel was run at 250V (linear) for 20 min and 4000V (linear) for 2hrs and finally at 4000V (rapid) for 10,000V hours. The IPG strip was taken out, mineral oil was removed and PAGE run was conducted to determine the exact molecular mass. The results were interpreted using the Biorad software.

MALDI/TOF MS Analysis

Matrix assisted laser desorption ionization mass spectrometer with TOF (Time-of-flight) is a soft ionization technique used in mass spectrometry. It allows the analysis of biomolecules such as proteins,

peptides and sugars which tend to be fragile. The MALDI/TOF MS is widely used mainly due to its large mass range. To determine more accurate molecular weight of the protein, MALDI-MS detector (Kratos Kompact MALDI II, England) was used. Protein mass identification is confirmed by MALDI-TOF/TOF.

UV-Vis analysis of purified laccase

The purified laccase UV-Vis (200-700nm) absorbance spectrum was taken using spectrophotometer (UV1700 Pharmaspec Shimadzu). Typical purified laccase exhibit characteristic absorbance maxima around 280nm and 600nm and a shoulder near 330nm. The ratio of the absorbance of pure laccase at 280nm to that at 600nm is generally 14 to 30 and the ratio of the absorbance at 330nm to that at 600nm is 0.5 to 2 (Wahleithner *et al.,* 1996; Xu *et al.,* 1996; Yaver *et al.,* 1996).

Enzyme Kinetics

The Km and Vmax of the purified laccase was estimated by using ABTS as substrate. The analysis was done using sigmaplot software.

Effect of pH on purified laccase

The purified laccase was incubated in buffers from pH 3 to 11. The activity was determined after 1hr and 24hrs of incubation.

Effect of temperature on purified laccase

The purified enzyme was incubated at different temperatures ranging from 30 to 80°C for 1 hr and activity was measured.

Effect of inhibitors on purified laccase

The enzyme was preincubated for 15min in the presence of different concentrations of laccase inhibitors like EDTA, sodium azide (NaN3), Thioglycolic acid (TGA), dithiothreitol (DTT) and L-cys. The effect of inhibitors on the activity was measured.

Effect of metal ions on purified laccase

The purified enzyme was incubated with 5mM concentration of the metal ions like Mn2+, Mg2+, Na+, Ca2+, Fe2+, Zn2+ and Cu2+ for 5 min and the activity was measured.

Effect of detergents on purified laccase

The 4 detergents selected for the study are SDS, Triton X 100, Tween 20 and Tween 80. The purified laccase was incubated for 10min with 1% detergent solution and the activity was measured.

Effect of solvents on purified laccase

Purified laccase was incubated with solvents like ethanol, acetone, acetonitrile, chloroform, DMSO, ethyl acetate, formamide and methanol. The activity was estimated after incubating the enzyme in 1% detergents for 1 hour.

Effect of NaCl on purified laccase

Halo tolerance of purified laccase was studied by incubating the purified laccase in different concentrations of NaCl (0.001M, 0.005M, 0.01M, 0.05M, 0.1M, 0.5M and 1M).

RESULTS AND DISCUSSION

Production of laccase enzyme

Production of Arthrographis KSF_2 laccase

The laccase production of Arthrographis KSF_2 in VMM is represented in Fig. 1. The logarithmic increase in specific activity is noted and it was noted that the maximum production of enzyme was in the 25th day of inoculation. The laccase producing potential of the specific strain of Arthrographis was earlier demonstrated by the authors in their previous study. (Devasia, S. and Nair, A. J., 2016b).

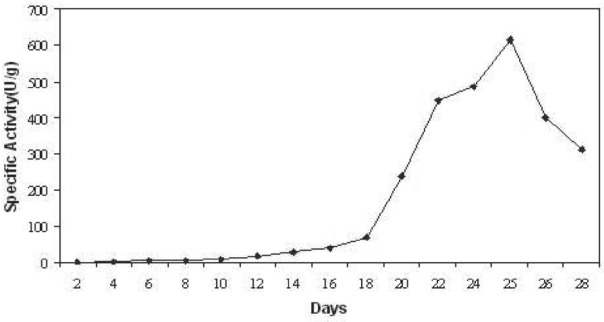

Fig. 1. Specific activity of the laccase enzyme

Purification of *Arthrographis* KSF_2 laccase

In fractional precipitation of the filtrates majority of protein was precipitated in 60% and 80% fractions. The pellet of each fraction was dissolved in 1ml 0.1M citrate buffer pH 5 and the specific activity of the fractions was 7.47 U/mg.

Analysis of the isoenzyme pattern of *Arthrographis* KSF_2 laccase

Native PAGE under non denaturing conditions was

conducted and on keeping the gel for 5-7 minutes in 1mM guaiacol, reddish brown bands were developed in the gel. The organism produced 5 constitutive isoenzymes of laccase in the modified VMM (Fig. 2).

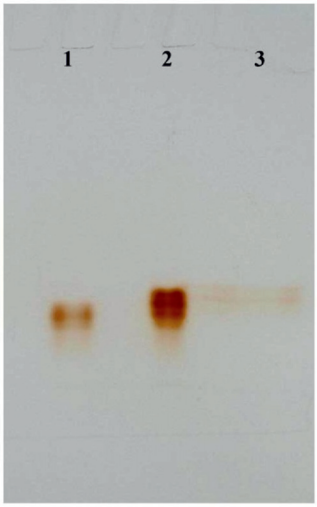

Fig. 2. Native PAGE showing Isoenzymes of Laccase

The major part of the laccase enzyme got precipitated in the 60% and 80% fractions. According to Palmieri *et al*. (1997) two laccase isoenzymes (POXA1 and POXA2) was produced by *Pleurotus ostreatus* with molecular weight of 61 and 67 kDa, pI of 6.7 and 4, respectively. Four laccase isozymes (LCC1, LCC2, LCC3 and LCC4) synthesized by *Pleurotus ostreatus* strain V-184 were purified and characterized (Mansur *et al*., 2003). LCC1 and LCC2 have molecular masses of about 60 and 65 kDa and exhibited the same pI value (3.0). Laccases LCC3 and LCC4 were characterized by SDS-PAGE, estimating their molecular masses around 80 and 82 kDa, pI 4.7 and 4.5, respectively. When staining with ABTS and guaiacol in native polyacrylamide gels, different specificities were observed for LCC1/LCC2 and LCC3/LCC4 isozymes. Cordi *et al*. (2007) extracted two isoenzyme forms of laccase from *Trametes* sp. Three laccase isoenzymes Lac I, Lac II and Lac III from *C. unicolor* had significantly varying biochemical characteristics (D'Souza-Ticlo *et al*., 2009).

Ion Exchange Chromatography (IEC)

The concentrated protein sample (100µl) was loaded and was allowed to move down the DEAE matrix at a flow rate of 1-1.5 ml/min. The protein elution pattern was monitored from the IEC chromatogram

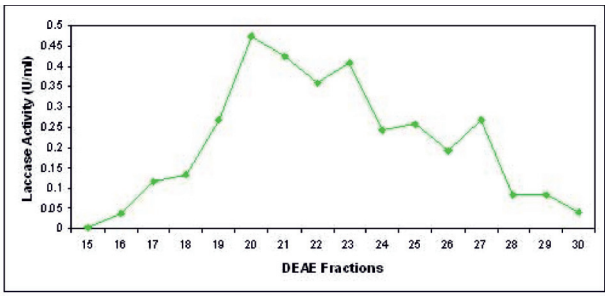

Fig. 3. ABTS assay of DEAE fractions

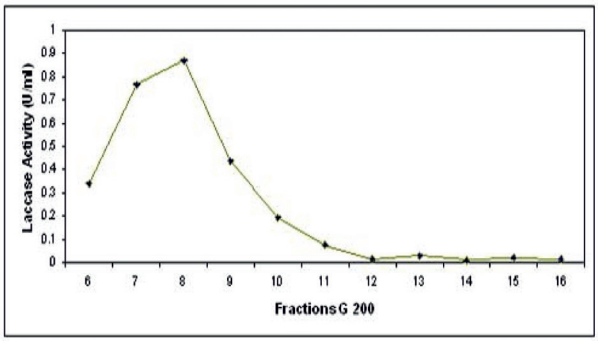

Fig. 4. ABTS assay of G 200 fractions

(Fig. 3). The selected samples were taken for ABTS assay and it was observed that the protein started eluting from the 19th fraction to the 25th fraction. Major proportion of the laccase enzyme was eluted in the 0.01M pH 8.2 buffer with 0.03-0.04M NaCl.

A series of DEAE columns were run, the fractions containing maximum laccase activity was pooled and concentrated by ultrafiltration. The active enzyme was subjected gel permeation chromatography (GPC).

Gel Permeation Chromatography (GPC)

Sephadex G 200 GPC column was carried out (Exclusion limit 5-600KD). G200 resin was filled in a longer glass column so that maximum retention time was allowed by adjusting the flow rate. ABTS assay of the fractions was done and it was observed that laccase stated eluting from the 6th fraction (Fig. 4).

The 8th fraction was with maximum activity and least protein, hence this fraction was selected as the purest fraction obtained after the series of chromatographic techniques employed. UF of the sample was conducted and the purity was confirmed by SDS PAGE (Fig. 5). Zymogram analysis was also done to show the activity of the purified laccase, Lac 4 (Fig. 6). Protein estimation was done by Lowry's method and the specific activity of the purified protein is explained in Table 1.

Only 24% of the total laccase activity was contributed by Lac 4, so the total yield of Lac4 from the crude filtrate was only 3.43%. When only the Lac4 is taken into account there was 14.19% yield after the purification protocol. Percentage of the yield of the enzyme after each purification step and purification fold of the enzyme was calculated.

Fig. 5. SDS PAGE of *Arthrographis KSF*$_2$ laccase

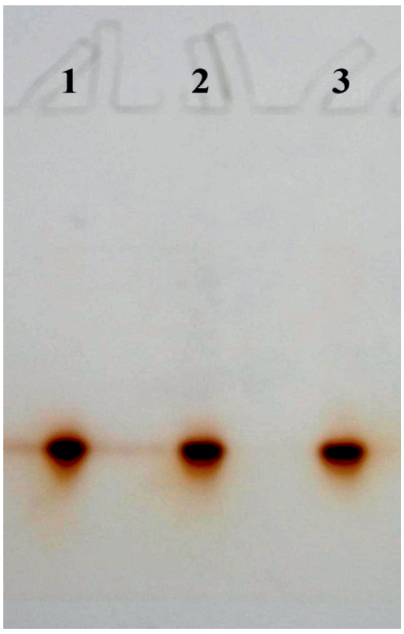

Fig. 6. Zymogram analysis of purified Lac4

Two Dimensional Polyacrylamide Gel Electophoresis

2D gel was under denaturing conditions using a 7cm IPG strip of 3-10 pI followed by SDS PAGE analysis. The pI of the protein was estimated as 5.5 and the molecular weight was estimated as 55KD (Fig. 7).

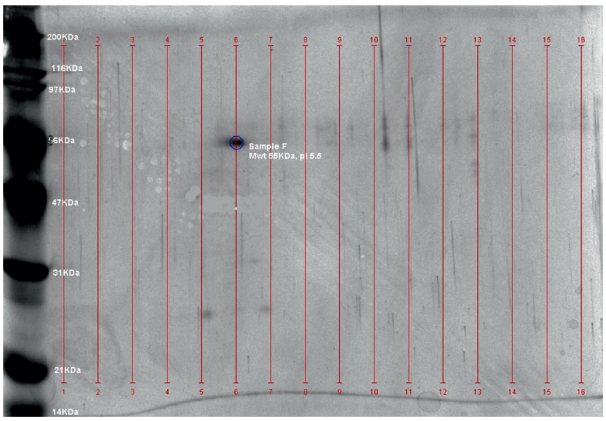

Fig. 7. 2D Analysis of *Arthrographis KSF*$_2$ laccase

Fungal laccases have pI ranging from 3-7. Lac IId (isozyme of laccase from *Cerrena unicolor*) purified by D'Souza-Ticlo *et al*. (2009) showed a molecular weight of 59 kDa and pI of 5.3 when analyzed by 2-D PAGE. The relative molecular masses of LacI, LacII and LacIII from *Trametes* sp. pwere reported approximately 64.2, 60.7 and 38.9 kDa and pI values of 7.3, 4.7 and 3.5 respectively (Weiyun *et al*., 2012).

MALDI/TOF MS Analysis

Matrix assisted laser desorption ionization mass spectrometer with TOF (Time-of-flight) is a soft ionization technique used in mass spectrometry. It allows the analysis of biomolecules such as proteins,

Table 1. Purification of laccase from *Arthrographis KSF*$_2$

Purification steps	Total Enzyme Activity (U)	Total Protein content (mg)	Specific Activity (U/mg)	Yield %	Purification fold
Crude	8671	680	12.75	100%	
Ammonium Sulphate (60%)	2090	136.6	15.3	24.1% (Lac 4)	1.2
DEAE & UF	973.39	5.97	163	11.23	12.78
GPC & UF	296.6	1.41	210.35	3.42	16.5

peptides and sugars which tend to be fragile. The MALDI/TOF MS is widely used mainly due to its large mass range. To determine more accurate molecular weight of the protein, MALDI-MS detector (Kratos Kompact MALDI II, England) was used. Protein mass identification was confirmed by MALDI-TOF/TOF. The Maldi analysis showed a strong peak at 55,140 Da (Fig. 8).

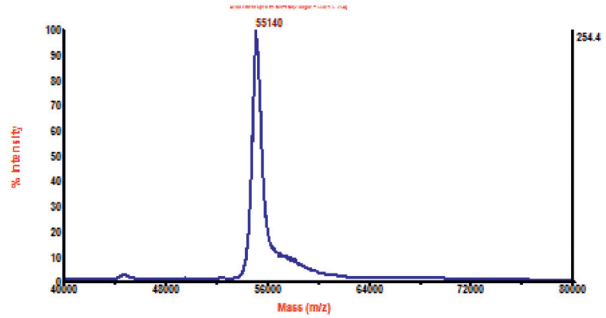

Fig. 8. Determination of molecular mass by MALDI-TOF/TOF

The molecular weights of laccases from fungi lie in the range of 40-80 kDa. The mass spectrometric determination for the large molecular weight molecules are common and MALDI/TOF MS system can be used to determine the accurate molecular weight. Kim *et al.* (2002) described the mass spectrum of laccase with a strong parent peak at m/z = 57,608 and they explained that the peak at 29,005 is from doubly charged parent molecule.

UV Analysis

UV analysis of the purified laccase was carried out and the ratio of the absorbance was analysed. The purified laccase on UV-Vis analysis did not show the characteristic laccase peak at 600nm and so it was confirmed as a typical yellow laccase. The ratio of absorbance at 280 to 600 was 14 and the ratio of absorbance of 330 to 600 was 4.5. The purity ratio analysis results indicated that the laccase obtained by the purification techniques was very close to 100% purity. The absorbance ratio at 280/600 is considered as the purity standard of laccase.

The occurrence of laccase which lack the typical absorption around 600nm has been reported in *Pleurotus* (Leontievsky *et al.*, 1997; Palmieri *et al.*, 1997). In this enzyme, the copper may be in the reduced form. Laccases from solid-state cultures of *Panus tigrinus, Phlebia radiata* and *Agaricus bisporus D-649* (Faure *et al.*,1994) grown on wheat straw were yellow-brown and had no typical blue oxidase spectra.

Characterisation of *Arthrographis* KSF_2 laccase

Enzyme Kinetics

The Km and Vmax of the purified laccase was estimated by using ABTS as substrate. The analysis was done using sigmaplot software. The enzyme showed a Km value of 15µM and Vmax of 21.9 µmol/min with ABTS as substrate (Fig. 9.a and 9.b). It was observed that the purified laccase obeyed Michaelis Menten and Lineweaver Burk kinetics.

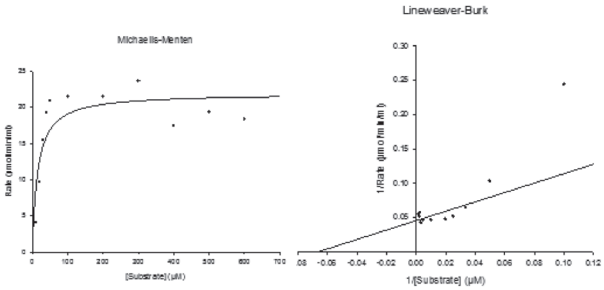

Fig. 9.a and 9.b. Enzyme kinetics studies

Effect of pH on purified laccase

The purified laccase was incubated in 0.1M buffers from pH 3 to 11. The activity was determined after 1hr and 24hrs of incubation. The enzyme was active in the alkaline pH range with pH optima at 5 and 11 and it was observed that there was 74.4% retention of activity at pH 11 after 24hrs (Fig. 10).

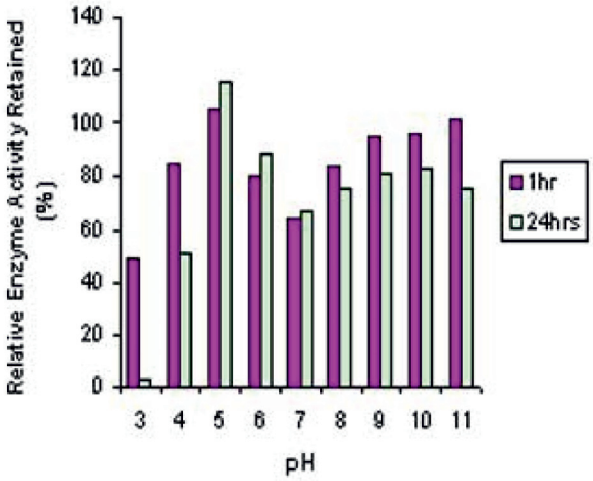

Fig. 10. Effect of pH on purified laccase

In pH 3 the purified laccase was found to be the least stable. The enzyme had high stability at pH 5 where it showed a 15% increased activity at 24hrs. It was surprising to see that the purified enzyme was also stable at extreme alkaline conditions.

The pH optima of laccases are highly dependable on the substrate. When using ABTS as substrate the pH optima are more acidic and are found in the range 3.0-5.0 (Heinzkill *et al.*, 1998). In general, laccase activity has a bellshaped profile with an optimal pH that varies considerably. This variation may be due to changes in the reaction caused by the substrate, oxygen or the enzyme itself (Xu, 1997). The difference in redox potential between the phenolic substrate and the T1 copper could increase oxidation of the substrate at high pH values, but the hydroxide anion (OH-) binding to the T2/T3 coppers results in an inhibition of the laccase activity due to a disruption of the internal electron transfer between the T1 and T2/T3 centres. These two opposing effects can play an important role in determining the optimal pH of the bi-phasic laccase enzymes (Xu, 1997). Laccase produced by *Trametes modesta* was fully active at pH 4.0 and very stable at pH 4.5 but its half-life decreased to 125 min at pH 3.0 (Nyanhongo *et al.*, 2002).

Effect of Temperature on purified laccase

An incubation of 1hr at 50 °C activates the enzyme by 5.49 %. Thus 50 °C was found to be the optimum for purified laccase. 99% activity was retained after 40 °C incubation for an hr. It was observed that at 60 °C there was a decrease of 31% activity and at 70 °C there was a decrease of 93.7% activity. The enzyme got 99.4% denatured at 80 °C (Fig. 11).

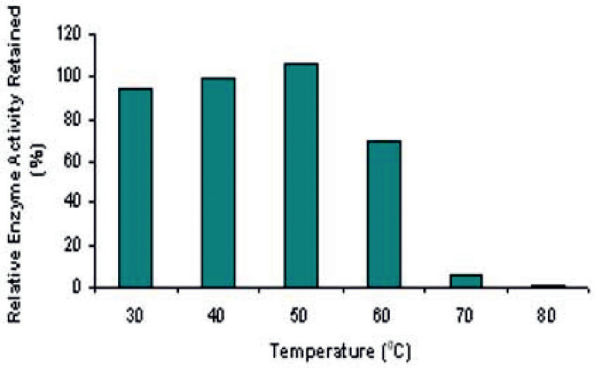

Fig. 11. Effect of temperature on purified laccase

Chefetz *et al.* (1998) proved that a preincubation of laccase for 2 min at 50 °C activated the enzyme. Temperature stabilities of laccases vary considerably, depending on the source organism. Laccases are stable at 30-50 °C and rapidly lose activity at temperatures above 60 °C (Wood 1980; Xu *et al.*, 1996; Chefetz *et al.*, 1998; Heinzkill *et al.*, 1998; Schneider *et al.*, 1999; Galhaup *et al.*, 2002a; Jung *et al.*, 2002; Palonen *et al.*, 2003). Farnet *et al.* (2000) further found that pre-incubation of enzymes at 40 °C and 50 °C greatly increased laccase activity. The laccases isolated from a strain of *Marasmius quercophilus* by Farnet *et al.* (2000) was found to be stable for 1 h at 60 °C. The laccase from *P. ostreatus* is almost fully active in the temperature range of 40 °C-60 °C, with maximum activity at 50 °C. The activity remains unaltered after prolonged incubation at 40 °C for more than 4 h (Call and Mucke, 1997). Nyanhongo *et al.* (2002) showed that laccase produced by *T. modesta* was fully active at 50 °C and was very stable at 40 °C but half-life decreased to 120 min at 60 °C. The most thermostable laccases have been isolated from bacteria; the half-life of *Streptomyces lavendulae* laccase was 100 minutes at 70 °C (Suzuki *et al.*, 2003) and that of *Bacillus subtilis* CotA was 112 minutes at 80 °C (Martins *et al.*, 2002). The typical half-lives of fungal laccases are clearly below one hour at 70 °C and below 10 minutes at 80 °C (Wood, 1980; Nishizawa *et al.*, 1995; Xu *et al.*, 1996; Chefetz *et al.*, 1998; Schneider *et al.*, 1999; Galhaup *et al.*, 2002a; Jung *et al.*, 2002; Palonen *et al.*, 2003).

Laccase enzyme extracted from *Trametes versicolor* by Han *et al.* (2005) exhibited high enzyme activity over broad pH and temperature ranges with optimum activity at pH 3.0 and a temperature of 50 °C. Laccase purified from *Stereum ostrea* found to be active and stable at optimal pH 6.0 and temperature 40 °C (Valeriano *et al.*, 2009).

Effect of inhibitors on purified laccase

The preincubation of pure laccase for 15min in EDTA, NaN_3, TGA, DTT and *L*-cys showed a decrease in enzyme activity. Laccase was inhibited partially or completely by the putative laccase inhibitors like EDTA, DTT, *L*-cysteine and NaN_3. (Fig. 12).

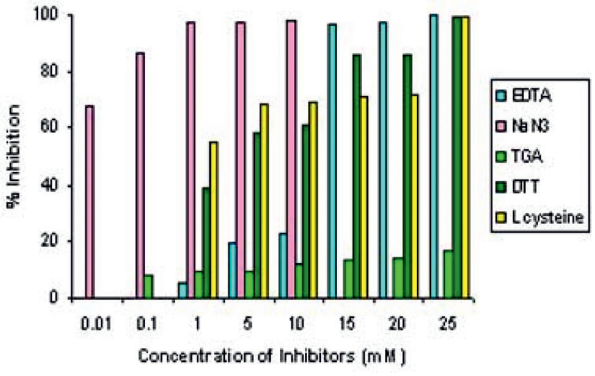

Fig. 12. Effect of inhibitors on purified laccase

There was 68% inhibition even by 0.01mM NaN_3 whereas the same concentrations of the other inhibitors had no effect on the enzyme activity. When the concentration was increased to 0.1mM the inhibition increased by 20% in the case of NaN_3. There was 8% inhibition by 0.1mM TGA whereas only 17% was inhibited by 25mM of the inhibitor. All the other inhibitors gave a 100% inhibition at 25mM concentration. A maximum of 99% inhibition was attained by NaN_3 at 10mM concentration.

Copper containing laccases are inhibited by azide and cyanide because they can bind to the type 2 and type 3 copper, resulting in an interruption of the internal electron transfer and activity inhibition (Gianfreda *et al*. 1999). It was reported by Niladevi *et al*. (2008) that complete inhibition of *Streptomyces psammoticus* laccase activity was observed with 0.1M NaN_3 and DTT whereas 100% inhibition by EDTA was observed at 2mM. Saito *et al*. (2003) have reported that sodium azide exerted little inhibition on laccase from a fungal strain at a concentration of 10mM while EDTA had no effect on the enzyme even at a very high concentration of 25mM. Many sulfhydryl-containing compounds such as *L*-cysteine, dithiothreitol and thioglycolic acid are also considered as laccase inhibitors. The inhibition was caused by reduction of the oxidized substrate by the sulfhydryl compounds and not by inhibition of the enzyme (Johannes and Majcherczyk, 2000). Dube *et al*. (2008) observed that EDTA (5 mM) totally inhibits laccase activity in *Streptomyces coellicolor*. Valeriano *et al*., 2009 reported that EDTA and SDS have prominent inhibitory effect on *Pynocorpus anguineus* laccase activity. Among the various inhibitors tested by D'Souza-Ticlo *et al*. (2009) for Lac IId, isozyme (isozyme of laccase from unicolor), activity was inhibited in the presence of sodium azide, SDS and mercaptoethanol.

Effect of metal ions on purified laccase

The effect of 5mM metal ions such as Mn^{2+}, Mg^{2+}, Na^{+}, Ca^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+} was estimated after incubating the enzyme with the metal ion for 5 min. It was observed that $FeCl_3$ inhibited the laccase activity to the maximum (70.5%) whereas the enzyme was least inhibited by $MnSO_4$ (21%). It was obsevered that even $CuSO_4$ inhibited the laccase activity in 5mM concentration (Fig. 13). The heavy metals are known laccase inhibitors, but here the inhibition by $FeCl_3$ was found to be higher.

It was reported by Niladevi *et al*. (2008) that the metal ions Fe, Cu, Zn, Na, and Mg (each at 2mM)

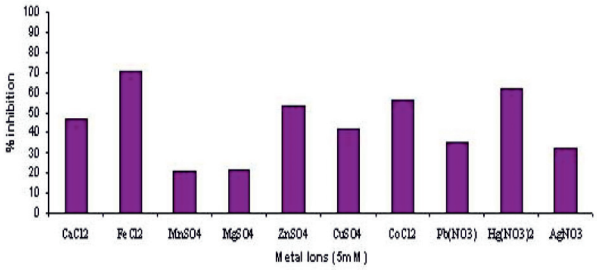

Fig. 13. Effect of metal ions on purified laccase

enhanced the enzyme activity whereas the heavy metals like Hg, Cd, Co, and Ni reduced the activity considerably even at 1mM concentration. Fe enhanced the laccase activity 2 fold and significant enhancement in enzyme activity was observed with Na, Cu and Zn. D'Souza-Ticlo *et al.* (2009) reported that the metal ions Sn, Ag and Hg inhibited the laccase activity in a marine- derived fungus by 32-37%.

Effect of detergents on purified laccase

The enzyme was incubated with the detergent solutions (SDS, Triton X 100, Tween 20 and Tween 80) and the activity was measured. There was 100% denaturation by 1% SDS in 10 min where as only 8% denaturation was observed by Tween 20. Tween 80 denatured 21% enzyme and Triton X 100 denatured 25% of the total enzyme (Fig. 14).

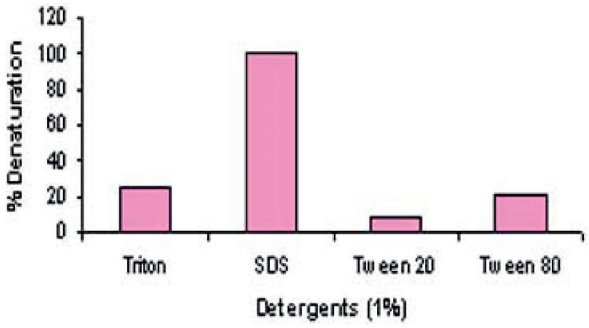

Fig. 14. Effect of detergents on purified laccase

Sunil *et al.*, (2011) reported that at 0.1% SDS concentration, complete laccase activity of *Pleurotus* sp was lost and hence he reported SDS as the most effective denaturant.

Effect of solvents on purified laccase

Purified laccase was incubated with 10% solvents and the activity was estimated after incubating the enzyme for 1 hour. It was observed that some of the solvents enhanced the enzyme activity whereas some denatured the pure enzyme. The enzyme was

least stable in the presence of ethyl acetate whereas the presence of alcohol enhanced the activity. Maximum enhancement of enzyme activity was in the presence of methanol (58%) and ethanol (29%). Acetone and DMSO activated the pure laccase by 13% whereas acetonitrile and formamide enhanced the activity by 8%. Chloroform inhibited the enzyme by 21% and ethyl acetate by 50% (Fig. 15).

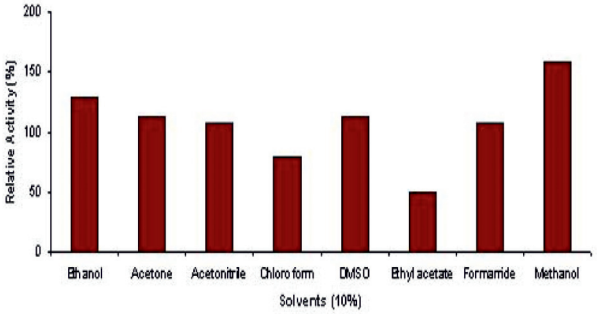

Fig. 15. Effect of solvents on purified laccase

Singh *et al.,* (2009) reported that the laccase from gamma-proteobacterium JB retained 80-100% activity in 10% concentration of dimethylsulfoxide (DMSO), ethanol, acetone or methanol. Zhao *et al.*, (2012) reported that the purified laccase from the deuteromycete fungus *Myrothecium verrucaria NF-05* retained approximately 80% of its initial activity in the presence of 5% methanol and ethanol. However, the inhibitory effect increased with the increasing concentration of solvents. The activity was almost completely inhibited in the presence of 20% acetone and acetonitrile. The activity was completely inhibited when the concentration of all of the tested solvents increased to 50%.

The present results indicated that the *Arthrographis KSF*$_2$ laccase enzyme might be suitable for use in reactions that require a similar concentration of these solvents.

Halo tolerance of purified laccase

This parameter was studied by incubating the purified laccase in different concentrations of NaCl (0.001M, 0.005M, 0.01M, 0.05M, 0.1M, 0.5M and 1M). Low concentrations of NaCl (1mM and 5mM) had an inductive effect on pure laccase and as concentration increased to 0.01M there was 43% inhibition by the salt. The increase in inhibition was directly proportional to the increase in salt concentration. When the concentration increased to 1M there was 100% inhibition by NaCl (Fig. 16).

Singh *et al.,* 2009 reported that the laccase from gamma-proteobacterium JB was not inhibited even

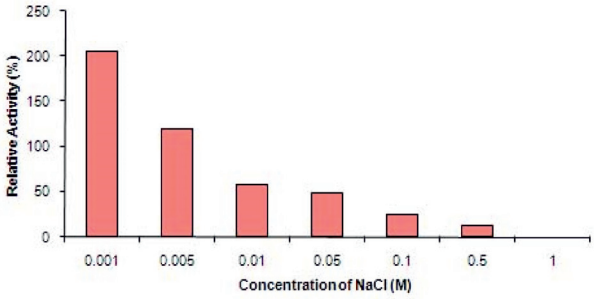

Fig. 16. Effect of NaCl on purified laccase

in the presence of 0.5 M NaCl. Pure sodium chloride up to 0.3 M, did not inhibit *Cerrena unicolor* MTCC 5159 laccase above which, the laccase was reversibly inhibited (D' Souza *et al*., 2009).

CONCLUSION

The total laccase activity of *Arthrographis KSF*$_2$ was found to be contributed by 6 isoenzymes. The major activity of *Arthrographis KSF*$_2$ was contributed by Lac4 and hence this isozyme was purified to homogeneity. The pI of Lac4 was estimated as 5.5 and the molecular weight was estimated as 55KD. The fungal laccase was stable at alksline pH and was stable at 50°C. The *Arthrographis KSF*$_2$ laccase was a typical laccase active at alkaline pH and the molecular mass and pI was of typical fungal laccase. The Laccase was inhibited partially or completely by the putative laccase inhibitors like EDTA, DTT, *L*-cysteine and NaN_3. All the other inhibitors except TGA gave almost 100% inhibition at 25mM concentration. On analysis of its halotolerant property it was observed that the increase in inhibition was directly proportional to the increase in salt concentration. The purified laccase on UV-Vis analysis did not show the characteristic laccase peak at 600 nm and so it was confirmed as a typical yellow laccase. Due to its highly active nature at alkaline pH and its tolerance to other solvents, chemicals and salt concentrations, it can find applications in industries applying extreme conditions.

Author contributions

SD and AJN has done the conceptualization; SD has done Data curation and Formal analysis; AJN has done Funding acquisition; SD and AJN has done Investigation; Methodology development and Project administration; AJN has done Supervision and Validation; SD has prepared manuscript and AJN has reviewed and approved the manuscript.

Funding

This work was supported by the Kerala University PhD fellowships.

Declarations of interest: None

REFERENCES

Archibald, F.S., Bourbonnais, R., Jurasek, L., Paice, M. G., and Reid, I.D.1997. Kraft pulp bleaching and delignification by *Trametes versicolor*. *Journal of Biotechnology.* 53: 215-236.

Assavanig, A., Amornkitticharoen, B., Ekpaisal, N., Meevootisomand Flegel, T.W. 1992. Isolation, characterization and function of laccase from *Trichoderma*. *Appl.Microbiol. Biotechnol*. 38: 198-202.

Call,H.P. and Mucke,I.1997. History, overview and applications of mediated lignolytic systems, especially laccase mediator systems (Lignozym®-process). *J. Biotechnol*. 53: 163-202.

Chefetz, B., Chen, Y. and Hadar, Y. 1998. Purification and characterization of laccase from *Chaetomium thermophilum* and its role in humification. *Appl. Environ. Microbiol*. 64: 3175-3179.

Cordi, L., Minussi, R.C., Freire R.S. and Duran, N. 2007. Fungal laccase: copper induction, semi purification, immobilization, phenolic effluent treatment and electrochemical measurement. *Afr. J. Biotechnol.* 6: 1255-1259.

Devasia, S. and Nair, A.J. 2016a. Optimization of Cultural and Nutritional Parameters of Arthrographis KSF2 a novel strain of Ascomycetes for Enhanced Laccase Production. *J. Microbiol. Biotech. Res.* 6 (2): 6-14.

Devasia, S. and Nair, A.J. 2016b. Screening of Potent Laccase Producing Organisms Based on the Oxidation Pattern of Different Phenolic Substrates. *Int. J. Curr. Microbiol. App. Sci.* 5(5): 127-137.

D' Souza- Ticlo, D., Sharma D. and Raghukumar, C. 2009. A thermostable metal- tolerant laccase with bioremediation potential from a marine- derived fungus. *Mar. Biotechnology*. 11: 725-737.

Farnet, A.M., Criquet, S., Tagger, S., Gil, G. and Le Petit, J. 2000. Purification, partial characterization, and reactivity with aromatic compounds of two laccases from *Marasmius quercophilus* strain 17. *Canadian Journal of Microbiology.* 46(3): 189-194.

Faure, D., Bouillant, M.L. and Bally, R. 1994. Isolation of *Azospirillum lipoferum* 4T Tn5 mutants affected in melanization and laccase activity. *Appl. Environ. Microbiol*. 60: 3413–3415.

Galhaup, C., Goller, S., Peterbauer, C.K., Strauss J. and Haltrich, D. 2002. Characterization of the major laccaseiso enzyme from *Trametes pubescens* and regulation of its synthesis by metal ions. *Microbiology.* 148: 2159-2169.

Gianfreda, L., Xu, F. and Bollag, J.M. 1999. Laccases: A useful group of oxidoreductive enzymes. *Bioremediation J.* 3: 1–25.

Han, M. J., Choi, H.T. and Song, H. G. 2005. Purification and characterization of laccase from the white rot fungus Trametes versicolor. *J. Mirobiol.* 43: 555-560.

Heinzkill, M., Bech, L., Halkier, T., Schneider P. and Anke, T. 1998. Characterization of laccases from wood-rotting fungi (family *Coprinaceae*). *Appl. Environ. Microbiol.* 64: 1601-1606.

Holker, U. and Lenz, J. 2005. Solid-state fermentation - are there any biotechnological advantages? *Curr. Opin. Microbiol*. 8: 301.

Johannes, C. and Majcherczyk, A. 2000. Laccase activity tests and laccase inhibitors. *J. Biotechnol*. 78: 193-199.

Jia Li Dong, 2004. Influence of culture conditions on laccase production and isoenzyme pattern in the white rot fungus Trametes gallica. *J. Basic Microbiol*. 45: 190-198.

Jung, H., Xu F. and Li, K. 2002. Purification and characterization of laccase from wood-degrading fungus *Trichophhyton rubrum* LKY-7. *Enz. Microb. Technol*. 30: 161-168.

Kim, Y., Cho, N.S., Eom, T.J. and Shin, W. 2002. Purification and characterization of a laccase from Cerrena unicolor and its reactivity in lignin degradation. *Bulletin of the Korean Chemical Society*. 23(7): 985–989.

Leontievsky, A.A., Myasoedova, N.M., Pozdnyakova, N.N. and Golovleva, L.A. 1997. 'Yellow' laccase of *Panus tigrinus* oxidizes non-phenolic substrates without electron-transfer mediators. *FEBS Letters*. 413: 446–448.

Mansur, M., Arias, M.E., Copa-Patino, J.L., Flardh, M. and Gonzalez, A.E. 2003. The white-rot fungus *Pleurotus ostreatus* secretes laccase isozymes with different substrate specificities. *Mycologia.* 95: 1013-1020.

Martins, L.O., Soares, C.M., Pereira, M.M., Teixeira, M., Costa, T., Jones G.H. and Henriques, A.O. 2002. Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. *J. Biol. Chem*. 277: 18849-18859.

Michniewicz, A., Ullrich, R., Ledakowicz, S. and Hofrichter, M. 2006. *Cerrena unicolor* strain 137 produces two laccaseisoenzymes with different physical and catalytic properties. *Applied Microbiology and Biotechnology.* 69: 682-688.

Niladevi, K. N., Nicemol, J. and Prema, P. 2008. Evidence for a halotolerant alkaline laccase in Streptomyces psammoticus: Purification and characterization, Process Biochemistry. 3: 654-660.

Nyanhongo, G.S., Gomes, J., Gubitz, G., Zvauya, R., Read J.S., and Steiner,W. 2002. Production of laccase by a newly isolated strain of Trametesmodesta. *Bioresource Technology*. 84(3): 259-263.

Nishizawa, Y., Nakabayashi, K. and Shinagawa, E. 1995. Purification and characterization of laccase from white rot fungus *Trametes sanguinea* M85-2. *J. Ferment. Bioeng*. 80: 91-93.

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capasso, A. and Sannia, G. 1997. A novel white laccase from *Pleorotusostreatus*. *J. Biol. Chem*. 272: 31301-31307.

Palonen, H., Saloheimo, M., Viikari, L. and Kruus, K. 2003.

Purification characterization and sequence analysis of a laccase from the ascomycete *Mauginiella* sp. *Enz. Microb. Technol.* 33: 854-862.

Ramamurthy, V. and Kothari, R.M. 1993. Comparison of fungal protease production by submerged and surface cultivation, *J. Biotechnol.* 27: 349-354.

Robinson,T., McMullan G. and Marchant, R. 2001. Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. *Bioresour. Technol.* 77: 247-255.

Saito, T., Hong, P., Kato, K., Okazaki, M., Inagaki, H., Maeda, S. 2003. Purification and characterization of an extracellular laccase of a fungus (family Chaetomiaceae) isolated form soil. *Enzyme Microb Technol.* 33: 520-526.

Sandhya, C., Sumantha, A., Szakacs, G. and Pandey, A. 2005. Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation. *Process Biochem.* 40: 2689-2694.

Schneider, P., Caspersen, M. B., Mondorf, K., Halkier, T., Skov, L.K., Ostergaard, P.R., Brown, K.M., Brown, S.H. and Xu, F. 1999. Characterization of a *Coprinus cinereus* laccase. *Enz. Microb. Technol.* 25: 502-508.

Singh, G., Sharma, P., Capalash, N. 2009. Performance of an alkalophilic and halotolerant laccase from gamma-proteobacterium JB in the presence of industrial pollutants. *J Gen Appl Microbiol.* 55(4): 283-289.

Singhania, R.R., Sukumaran R.K. and Patel, A.K., Larroche, C. Pandey, A. 2010. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. *Enzym. Microb. Tech.* 46: 541-549.

Sunil, S. More, Renuka, P. S., Pruthvi, K., Swetha, M., Malini, S. and Veena S. M. 2011. Isolation, Purification, and Characterization of Fungal Laccase from Pleurotus sp. Enzyme Research, Article ID 248735, 7 pages.

Suzuki, T., Endo, K., Ito, M., Tsujibo, H., Miyamoto, K. and Inamori, Y. 2003. A thermostablelaccase from *Streptomyces lavendulae* REN-7: purification, characterization, nucleotide sequence, and expression. *Biosci. Biotechnol. Biochem*. 67: 2167-2175.

Valeriano, V.S., Silva, A.M.F., Santiago, M.F., Bara M.T.F. and Telma, A.G. 2009. Production of laccase by Pynocorpus sanguineus using 2,5-xylidine and ethanol. *Braz. J. Microbiol.* 40: 790-794.

Wahleithner, J.A., Xu, F., Brown, K.M., Brown, S.H., Golightly, E.J., Halkier, T., Kauppinen, S., Pederson, A., Schneider, P. 1996. The identification and characterization of four laccases from the plant pathogenic fungus *Rhizoctonia solani. Curr Genet.* 29(4): 395-403.

Weiyun Guo, Zhaoyang Yao, Chenyan Zhou, Duan Li, Hongli Chen, Qiang Shao, Zongyi Li and Huigen Feng, 2012. Purification and characterization of three laccase isozymes from the white rot fungus *Trametes* sp. HS-03. *African Journal of Biotechnology.* 11(31): 7916-7922.

Wood, D.A. 1980. Production, purification and properties of extracellular laccase of *Agaricus bisporus. J. Gen. Microbiol.* 117: 327-338.

Xu, F. 1996. Oxidation of phenols, anilines and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. *Biochemistry*. 35: 7608-7614.

Xu, F. 1997. Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. *J. Biol. Chem.* 272: 924-928.

Yaver, D.S., Xu, F., Golightly, E.J., Brown, K.M., Brown, S.H., Rey, M.W., Schneider, P., Halkier, T., Mondorf, K. and Dalboge, H. 1996. Purification, characterization, molecular cloning, and expression of two laccase genes from the white-rot basidiomycete *Trametesvillosa. Applied and Environmental Microbiology* 62: 834–841.

Zhao, D., Zhang, X., Cui, D., Zhao, M. 2012. Characterisation of a Novel White Laccase from the Deuteromycete Fungus Myrothecium verrucaria NF-05 and Its Decolourisation of Dyes. PLoS ONE 7(6): e38817. doi:10.1371/journal.pone.0038817.