The Journal of Animal & Plant Sciences, 26(5): 2016, Page: 1399-1404
ISSN: 1018-7081

DELIGNIFICATION OF PAPER PULP BY PURIFIED LACCASE FROM *ASPERGILLUS FLAVUS*

M. S. Aslam[2], K. Hanif[2], S. U. Rehman[1], I. Gull[2], M. A. Athar[2] and Z. Abbas*[1]

[1]Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan
[2]Institute of Biochemistry and Biotechnology, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan
*Corresponding author Email address: zaigham.mmg@pu.edu.pk

ABSTRACT

The laccase enzyme was purified and characterized from the supernatant of the *Aspergillus flavus* (*A. flavus*) sprouting culture and its paper pulp delignification capacity was determined. The laccase production reached maximum level on 14th day of incubation in malt extract media at 40 ^{o}C. Specific activity and molecular mass of purified monomeric enzyme was found to be 780 U/mg and 40 *kDa* respectively. *Km* and *Vmax* values of the purified laccase were determined to be 12 µM and 3804 µM/min respectively. *A. flavus* laccase was strongly impeded by 5 mM L-cysteine, di-thiothreitol, Na-azide and moderately impeded by SDS and EDTA at concentration of 5 mM. Laccase activity was improved by Cu^{2+}, Ca^{+2} and Mg^{2+} ions while impeded by K^{+1}, Ni^{+2}, ,NH_4^{+1}, Ba^{+2} and Ag^{+2} at 1.0 mM concentration. The delignification capacity of extracellular laccase enzyme was examined by using 2, 2-azinobis-3-ethylbenzthiazoline-6-sulfonate as a mediator.The optimum pH and temperature for paper pulp delignification were established to be 4.5 and 40°C respectively while the optimum time was found to be 2 hours.

Key words: Laccase, Industrial Enzyme, Delignification, Paper Pulp.

INTRODUCTION

Laccases (EC 1.10.3.2) act as a biocatalyst and belongs to oxido reductases class of enzymes (Murugesan *et al.*, 2006). These are multiple Cu^{+2} incorporating enzymes that undergo oxidation of a number of phenol containing compounds along with reduction of O_2 to H_2O (Bourbonnais and Paice, 1992). Among a wide range of different oxidant enzymes that have been used in different industries, laccases have got much importance in the last decade due to their substrate specificity and efficiency to catalyze reactions at wide range of temperature and pH conditions.

Laccase is very thermostable enzyme and used in textile, pharmaceutical, food, chemical and wood processing industry. It's a very eco-friendly enzyme and also used in recycling of waste matter (Shraddha *et al.*, 2011). Chlorine and oxygen core chemicals are employed for the estrangement of lignin from paper pulp which is subsequently used for the production of paper on industrial scale. These chemicals are associated with drawbacks such as their heavy cost and recycling of toxic effluents like phenolic compounds that arise during lignin deterioration. Therefore laccase based delignification could be an alternative safe procedure in paper and pulp industry (Kunamneni *et al.*, 2007).

Lignocellulose is the chief constituent of dead plant matter, and the most lavish biomass on this planet earth. Lignin has uneven ordering of multiple units of phenyl-propanoid that can impede enzymatic or chemical deterioration to safeguard cellulose. Laccases have been thoroughly scrutinized, with special reference to lignin deterioration (Claus, 2003). Laccases extracted from fungus have elevated redox potential than the laccases extracted from bacterial or plant source. Therefore, fungal laccases are important for the deterioration of lignin. Production of paper on industrial scale is an energy requiring multi-step procedure, which makes large volumes of contaminants (Pokhrel and Viraraghavan, 2004). Consumption of laccase enzyme in pulp and paper manufacturing was explored in the recent past and its various useful implementations make this a good substituent of current expensive and harmful chemical processes (Barreca, *et al.*, 2003). The use of laccase in bio-pulping reduces the energy required for refining, enhance the strength of paper, improve yield and environment friendly.

In this study, production, screening, purification and characterization of extracellular laccase enzyme from *A. flavus* (White rot fungus) was investigated and its ability to delignify the paper pulp was evaluated.

MATERIALS AND METHODS

Screening of *A. Flavus* for Laccase production: *A. flavus* was acquired from Institute of Agricultural Sciences, University of the Punjab, Lahore. It was grown on a culture media containing peptone 0.1%, yeast extract 0.1%, agar 1.5% and starch 0.1% at 30 ^{o}C for 7 days and stored at 4 ^{o}C. Culture media to determine the activity of laccase enzyme was prepared with glucose 2%, peptone

1%, ABTS 0.02 %, malt extract 0.5% and $CuSO_4$ 0.0005%, and incubated at 30 ºC for 7 days.

Laccase Assay: 2,2-azinobis-3-ethylbenzthiazoline-6-sulfonate (ABTS) was employed as a mediator to observe enzyme activity from the sprouting culture of *A. Flavus*. Equal volume of substrate and culture was taken. 100µl of 10 mM ABTS, 100 µl enzyme sample and 800 µl of 50 mM Na-acetate buffer (5.0 pH) were stirred up and incubated at 30 ºC for 25 minutes. Oxidation of ABTS was checked at A_{420} (ϵ = 36,000 M/1cm/1) by spectrophotometer with the advent of green color. Productivity of enzyme was examined every day, starting from 3rd day to day 14 to reach the point of maximum laccase yield. Enzyme activity was determined in "Unit" where 1 unit of extracellular enzyme is the quantity of laccase required to oxidize 1 µM of its substrate (ABTS) per minute at 30 ºC temperature.

Purification of Laccase: *A. Flavus* was allowed to sprout for 14 days and culture media was filtered (using Whatman No.1 filter paper). Filtrate was spinned for 15 minutes at 6000 rpm, 4ºC and equal portion of ice cooled acetone was added in supernatant with continuous stirring on ice and was stow on ice for 2 hours. Then spinned at 10,000 rpm for 10 minutes at a temperature of 4 ºC and the pellet was dissolved into it 1ml of 0.05 M Na-acetate buffer of 5.0 pH. This mixture was dialyzed in 2L buffer solution of 0.05 molar Na-acetate buffer (pH 5.0). Bradford assay was done to estimate the total concentration of protein by taking BSA as a standard (Bradford, 1976). Purification of dialyzed enzyme was done by loading it on ion exchange chromatography column (1.5 cm × 20 cm) packed by DEAE-sephadex resin and equipoised by 0.05 M Na-acetate buffer. Enzyme was eluted in a step by step gradient set by 0.1, 0.2, 0.3, 0.4 and 0.5 M solution of NaCl in Na-acetate buffer, with a flow rate of 1.0ml/minute. Partially purified sample was loaded on sephadex G-100 (gel filtration chromatography) column (1.5 cm × 40 cm) packed by sephadex G-100 equipoised by 0.05 M Na-acetate buffer (pH 5.0). Laccase was eluted by gathering 2 ml fractions at a flow rate of 0.5 ml/minute. Lyophilization of fractions was done and the lyophilized enzyme was scrutinized by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Total concentration of purified enzyme was estimated by Bradford assay. Purified sample was again dialyzed in 2L Na-acetate buffer and was lyophilized, scrutinized by 10% SDS polyacrylamide gel electrophoresis and quantified by Bradford assay.

Characterization of Laccase: Purified enzyme was analyzed on 10% SDS Polyacrylamide gel electrophoresis (Laemmli, 1970). Coomassie Blue R-250 dye was employed to stain the gel and to make protein band conspicuous. Purified laccase enzyme along with 10 µM ABTS was incubated at 20ºC, 30ºC, 40ºC, 50ºC, 60ºC, 70ºC and 80ºC in Na-acetate buffer (pH 4.5) for 1 hour, to determine optimum temperature for its activity. Similarly optimum pH for its activity was calculated by using buffer solutions of different pH range; 50mM Na-acetate buffer of 2.0, 3.0, 4.0, 5.0 pH and Na-phosphate buffer of 6.0, 7.0, 8.0 pH. Fungal laccase was incubated with 10 mM 2,2-azinobis-3-ethylbenzthiazoline-6-sulfonate as mediator in 50 mM Na-acetate buffer of 4.0 pH in the presence 1mM K^{+1}, 1mM Mg^{+2},1mM Cu^{+2},1mM NH_4^{+1}, 1mM Ni^{+2}, 1mM Ca^{+2}, and 1mM Ag^{+1}separately to assess their effect on laccase activity.

Delignification of Paper Pulp by Laccase: Paper pulp was obtained from Packages Private Limited Lahore, to determine the delignification potential of purified laccase. Different aliquots with the concentration of 2mg/ml kraft pulp in distilled water were prepared and incubated with the enzyme in at 30ºC temperature for three hours. After incubation few drops of CCl_3COOH to the enzyme treated pulp and then its pH was adjusted to 9.0. The mixture was then subjected to centrifugation at maximum speed of 13000 rpm for 5 minutes. The supernatant was diluted 10 times and then its absorbance at 280nm was determined. The absorbance reflects the relative amount of lignin in the supernatant after delignification. The optimum pH for the delignification was estimated by incubating 2mg/ml of kraft pulp as the substrate and the purified laccase for three hours at 30ºC temperature with the mediator 2,2-azinobis-3-ethylbenzthiazoline-6-sulfonate in 50 mM Na-acetate buffer with different pH; pH 4, pH 4.2, pH 4.6, pH 4.8, and pH 5.2. Similarly, the optimum temperature for delignification of pulp was calculated by evaluating the absorbance at 280nm of laccase incubated pulp at different temperatures; 30ºC, 40ºC, 50ºC and 60ºC. Optimum time for delignification was measured by incubating the kraft pulp as substrate with the laccase for various time periods ranging from 1 hours to 5 hours in 50 mM Na-acetate buffer of 4.5 pH.

RESULTS

Screening Plates: *A. flavus* secretes laccase that was detected by adding 0.02 % ABTS as mediator in malt-extract-agar plate. Enzyme caused oxidation of ABTS is spotted by formation of green colored zones as shown in Figure 1A. Time period for yield of laccase by *A. flavus* in culture media was optimized. Results showed that the enzyme yield started in sprouting media on the day 7th and reached maximum on day 14 as shown in Figure 1B. The values are the mean of triplicates. Protein quantification of culture supernatant, acetone precipitation, ion-exchange and gel filtration chromatography purified laccase was done using Bradford assay and total protein concentration at each level of purification is given in Table 1.

Purification of Laccase: The laccase yield started in malt extract media on the 7th day and reaches an average peak level on day 14. Thus, the culture supernatant was estimated regularly for 14 days. Enzyme was purified from the sprouting culture supernatant of *A. flavus* by different chromatography techniques. Purification was started with the total protein precipitation by using ice chilled acetone. The pellet was mixed in 50 mM Na-acetate buffer of pH 4.0 and then scrutinized by 10% SDS Polyacrylamide gel electrophoresis as shown in Figure 2.

Fractions were collected from ion-exchange chromatography. Eluted fractions were analyzed spectrophotometrically with ABTS. Fractions 3 to 8 eluted at 0.25 M NaCl showed positive laccase activity. The positive fractions were dialyzed, concentrated by lyophilization and analyzed by 10% SDS polyacrylamide gel electrophoresis (Figure 2).

Partially purified enzyme was further purified by gel filtration chromatography by using sephadex G-100 column. Eluted enzyme fractions were analyzed spectrophotometrically with ABTS for the presence of laccase. Fractions were concentrated by lyophilization and were scrutinized by 10% SDS Polyacrylamide gel electrophoresis. The estimated molecular mass of active monomeric enzyme was 40 kDa.

Characterization of Purified Laccase: *A. flavus* laccase established maximum activity at pH 4 and temperature 40ºC (Figure 3). Metal ions effect on laccase activity was estimated by employing ABTS as mediator. The activity of *A. flavus* laccase was strongly impeded by 1mM of K^{+1}(95 % inhibition), Ni^{+2}(83 % inhibition) and Ag^{+1} (82% inhibition), and stimulated by Mg^{+2} (15% inhibition), and 1mM of Cu^{+2}, while unaffected by 1mM of $NH4^{+1}$ and Ca^{+2} (figure 4).

Paper Pulp Delignification: During delignification of paper pulp, as the lignin contents in the sample decreases, the absorbance of the sample increases, when observed at 280 nm (Brinkmann *et al.*, 2002). The delignification by *A. flavus* laccase was optimized at a pH 5.2 and temperature of 40ºC. The optimum time for delignification of paper pulp found to be 2 hours (figure 5).

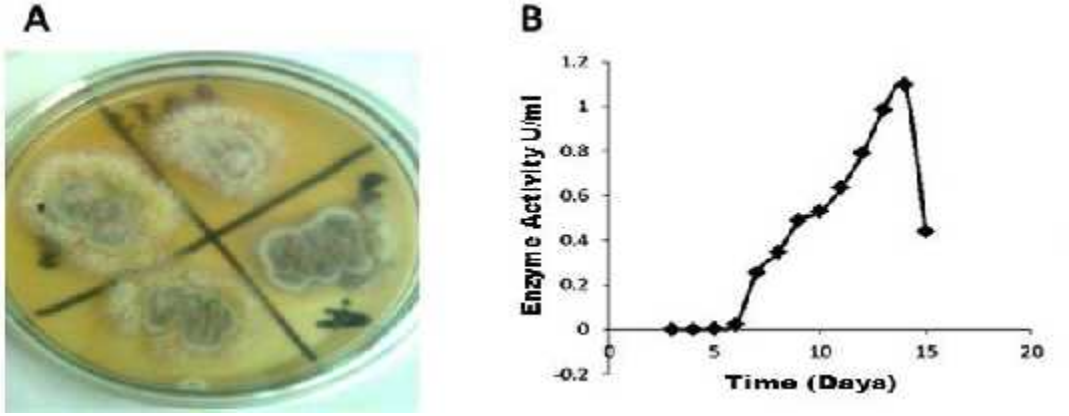

Figure 1. Growth of *A. flavus* and laccase production. (A) Oxidation of ABTS and formation of green color zones by laccase from *A. flavus* on Malt extract agar plate **(B)** Yield of laccase by *A. flavus* at different time points during sprouting in malt culture media.

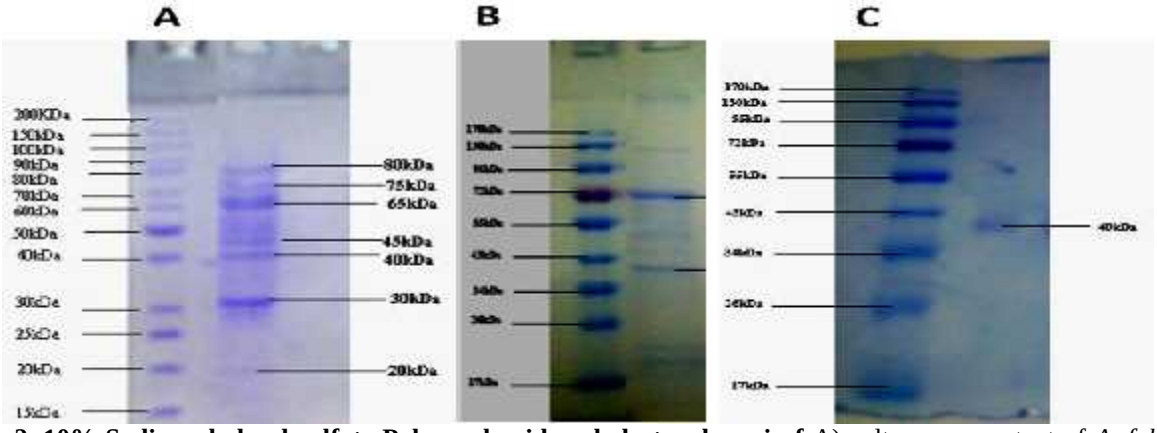

Figure 2. 10% Sodium dodecyl sulfate-Polyacrylamide gel electrophoresisof A) culture supernatant of *A. falvus* B) concentrated protein after acetone precipitation C) purified *A. flavus* laccase using gel filteration column (Sephadex G-100 column). **Lane 1** Marker, **Lane 2** Sample.

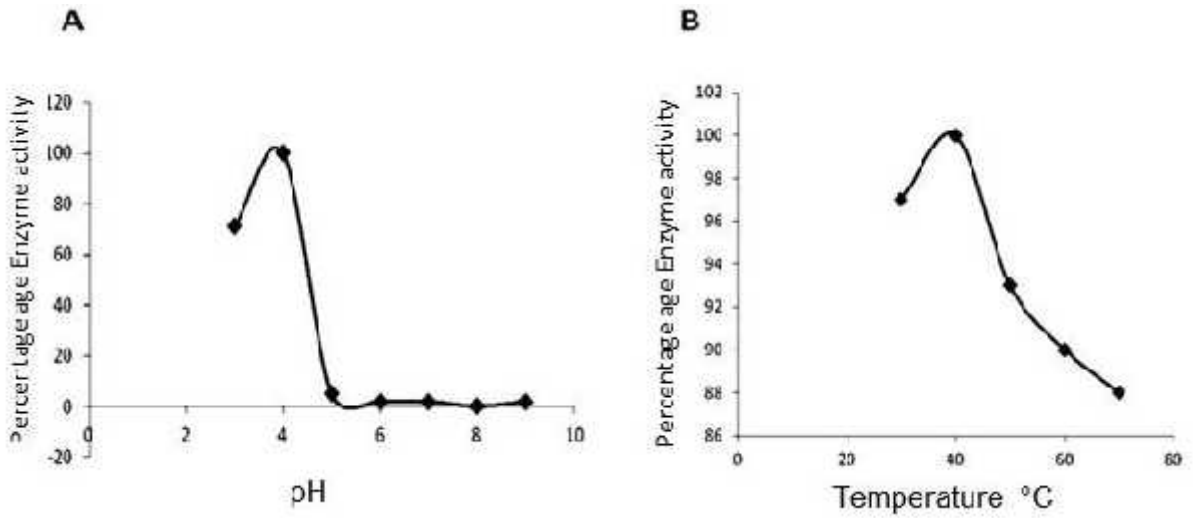

Figure 3. ***Optimum conditions for Laccase from A. Falvus.*** A) Optimum pH for best activity of *A. flavus* laccase . B) Optimum temperature for optimum activity of *A. flavus* laccase.

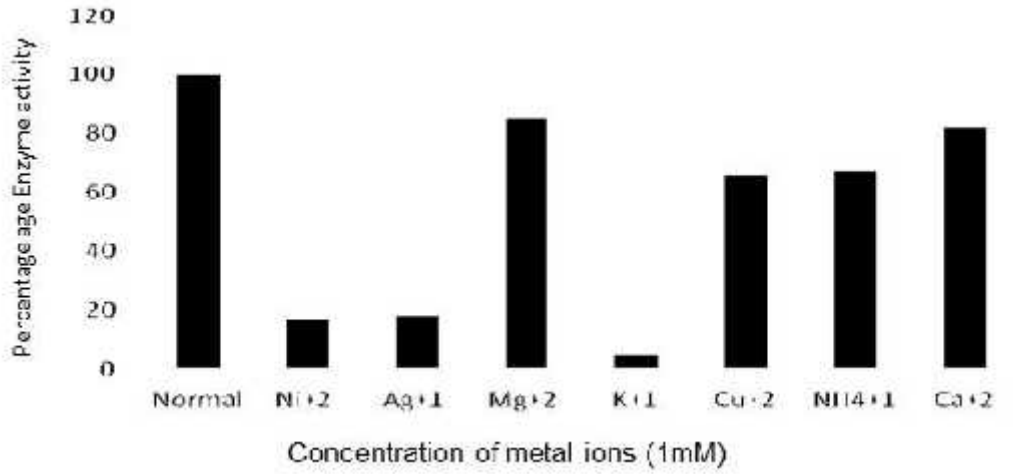

Figure 4. Antagonistic and synergistic effect of different metal ions on the activity of *A. flavus* laccase.

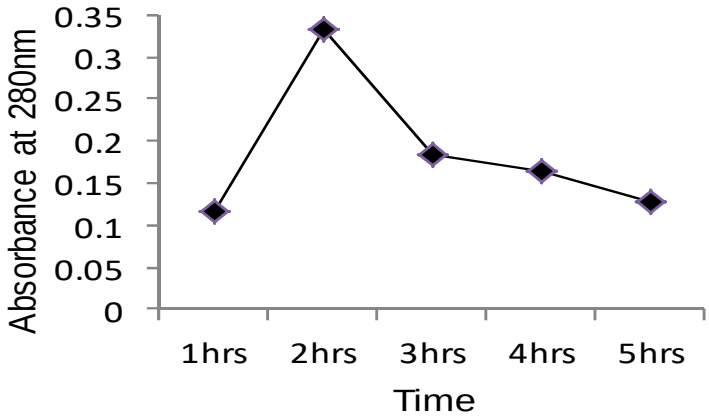

Figure 5 Identification of optimum incubation time required by *A. Flavus* laccase to delignify paper pulp.

Table1. Purification scheme of laccase from *A. flavus.*

Purification step	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Purification fold	Yield (%)
Culture supernatant	90	0.88	102.27	1	100
Acetone precipitation	82	0.51	160.78	1.57	91.11
DEAE-Cellulose	65	0.20	325	3.18	72.22
Sephadex G-100	78	0.10	780	7.63	86.67

Table 2. Molecular weight and Kinetic properties of *A. flavus* laccase.

Substrate	Molecular Weight (kDa)	Optimal pH	Optimal Temperature (ºC)	*Km* (µM)	*Vmax* (µM/min)
ABTS	40	4.5	40	12.0	3804

* Mean values of three experiments were given in above data

DISCUSSION

Laccases are the multiple Cu^{+2} incorporating enzymes which undergo oxidation of phenols, substituted phenols, aromatic amines, lignin, and polycyclic aromatic hydrocarbons (Bourbonnais and Paice, 1992). Fungal laccases are better to deteriorate lignin as they are not associated with potentially harmful phenolic substances which arise during chemical delignification methods methods (Claus, 2003).

The study was done to screen, isolate, purify and characterize *A. flavus* laccase. Screening plates were prepared by using ABTS as substrate in malt extract medium. Laccase secreted by *A. flavus* caused the oxidation of ABTS substrate which was present in the malt extract medium and formed green color colonies. Monomeric Laccase, extracted from the culture filtrate of *A. flavus* was purified by using different techniques such as acetone precipitation, anion exchange chromatography and gel filtration chromatography and have a molecular mass of 40 kDa as determined by SDS polyacrylamide gel electrophoresis. While, Laccases extracted from *Pleurotussp, Trametes versicolor* and *Cerrena sp.* have a molecular mass of 40± 1 kDa, 60 kDa and 58.6 kDa respectively (Minussi *et al.*, 2007; More *et al.*, 2011; Yang *et al.*, 2014).

A. flavus laccase enzyme was characterize using ABTS as substrate and optimum activity of the enzyme was found at 4.5 pH and 40°C temperatre. Our findings are in parallel to laccase extracted from different sources like *Gaeumannomyces graminis* and *Pleurotus sp.*which demonstrate optimum activity at 4.5 pH and 40 °C temperature. (Edens *et al.*, 1999; Minussi *et al.*, 2007; More *et al.*, 2011).

Metal ions effect the activity of extracellular laccase enzyme, in our study laccase enzyme was strongly impeded by 1mM of Ni^{+2} and Ba^{+2}, slightly impeded by Ag^{+1} and Mg^{+2}, slightly activated by 1mM of K^{+1} whlile remained unaffected by 1mM of Cu^{+2}, $NH^{+1}$and Ca^{+2}. In an earlier report $MgCl_2$, CuSO4, $ZnCl_2$ $FeCl_2$, $BaCl_2$ were found neutral for *Pleurotus sp*. laccases but $MnCl_2$ slightly decreased activity of laccases (More *et al.*, 2011). Similarly, in another study $Mn2^+$, Na^+, Mg^{2+},Ca^{2+}, Zn^{2+}, K^{+1} increase *Cerrena sp.* laccases activity, whereas Li^+ and Fe^{2+} decrease its activity (Yang *et al.*, 2014).

Laccases are very thermostable enzymes and have been tested for wide range of industrial applications. Laccases extracted from *Cerrena sp.* and *Trametestrogii* have very classic decolorization ability (Mechichi *et al.*, 2006). In our study the laccase enzyme was assessed for its delignification potential. The optimum pH and temperature for delignification of paper pulp was found 4.5 and 40°C respectively. The time required for maximum delignification was found to be 2 hours. The present study show that *A. flavus* laccase possess important delignification properties and could be used in paper pulp industry.

REFERENCES

Bourbonnais, R. and M. G. Paice (1992). Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate). Appl. Microbiol. Biotechnol. 36(6): 823-27.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72(1-2): 248-54.

Brinkmann, K., L. Blaschke and A. Polle (2002). Comparison of different methods for lignin determination as a basis for calibration of near-infrared reflectance spectroscopy and implications of lignoproteins. J. Chem. Ecol. 28(12): 2483-501.

Claus, H. (2003). Laccases and their occurrence in prokaryotes. Arch. Microbiol. 179(3): 145-50.

Edens, W. A., T. Q. Goins, D. Dooley and J. M. Henson (1999). Purification and characterization of a

secreted laccase of *Gaeumannomyces graminis* var. *tritici*. Appl. Environ. Microbiol. 65(7): 3071-74.

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

Minussi, R. C., M. A. Miranda, J. A. Silva, C. V. Ferreira, H. Aoyama, S. Marangoni, D. Rotilio, G. M. Pastore and N. Durán (2007). Purification, characterization and application of laccase from *Trametes versicolor* for colour and phenolic removal of olive mill wastewater in the presence of 1-hydroxybenzotriazole. Afr. J. Biotechnol. 6(10): 1248-1254.

More, S. S., R. PS and S. Malini (2011). Isolation, Purification, and Characterization of Fungal Laccase from *Pleurotus* sp. Enzyme Res. 2011: 1-7.

Murugesan, K., M. Arulmani, I.-H. Nam, Y.-M. Kim, Y.-S. Chang and P. T. Kalaichelvan (2006). Purification and characterization of laccase produced by a white rot fungus *Pleurotus* sajor-caju under submerged culture condition and its potential in decolorization of azo dyes. Appl. Microbiol. Biotechnol. 72(5): 939-46.

Pokhrel, D. and T. Viraraghavan (2004). Treatment of pulp and paper mill wastewater—a review. Sci. Total Environ. 333(1-3): 37-58.

Yang, J., Q. Lin, T. B. Ng, X. Ye and J. Lin. 2014. Purification and characterization of a novel laccase from *Cerrena sp*. HYB07 with Dye decolorizing ability. PLoS ONE. 9(10): 1-13.

Shraddha, R. S., S. Simran, K. Mohit, and K. Ajay (2011). Laccase: microbial sources, production, purification, and potential biotechnological applications. Enzyme Research, 2011: 1-11.

Barreca, A.M., M. Fabbrini, C. Galli, P. Gentili and S. Ljunggren (2003). Laccase/mediated oxidation of a lignin model for improved delignification procedures. J. Mol. Catal. B-Enzym; 26(1-2): 105-110.

Kunamneni, A., A. Ballesterose, F.J. Plou, and M. Alcalde (2007). Fungal laccase- A versatile enzyme for Biotechnological applications. Trends in Appl. Microbiol. 233-245.

Hela Zouari-Mechichi , Tahar Mechichi, Abdelhafidh Dhouib, Sami Sayadi, Angel T. Martınez, Maria Jesus Martinez, Laccase purification and characterization from *Trametes trogii* isolated in Tunisia: decolorization of textile dyes by the purified enzyme, Enzyme and Microbial Technol. 39(2006): 141–148.