Supported by tetfund TERTIARY EDUCATION TRUST FUND

PRODUCTION AND PARTIAL CHARACTERIZATION OF pH AND METALLO-STABLE LACCASE OF *TRAMETES* SP. G31 WITH INDUSTRIAL POTENTIALS

[1]*Benjamin Vandelun Ado, [2] Etim Etim Esin and [3] Muinat Olanike Kazeem
[1]Department of Microbiology, College of Biological Sciences, Joseph Sarwuan Tarka University, Makurdi, Nigeria.
[2]Department of Biochemistry, College of Biological Sciences, Joseph Sarwuan Tarka University, Makurdi, Nigeria.
[3]Department of Microbiology, Faculty of Life Sciences, University of Ilorin, Kwara State, Nigeria
***Correspondence E-mails:** adobenjamin2014@gmail.com, benjaminado@yahoo.com.

Received: December 13, 2021 Accepted: February 20, 2022

Abstract

Laccases catalyze a vast range of substrates owing to low substrate specificity. However, for large-scale applications, laccases with high activity and stability are crucial. In the study, laccase and Total Soluble Protein (TSP) from *Trametes* sp. isolate G31 was produced at pH 3.0 - 8.0 in solid state fermentation using saw-dust of *Terminalia superba*. A fraction of the crude enzyme was partially purified and further characterized. The optimum fermentation period for laccase and TSP production were day 14 (2113 U/mL) and day 6 (2.52 mg/mL) respectively. The pH optimum for laccase production ranged from pH 5.0 (2356 U/mL) - pH 7.0 (2369 U/mL) and TSP at pH 3.0 (2.50 mg/mL). Enzyme kinetics showed optimum activity at pH 3.0 - pH 5.0 (2032 U/mL), high stability at pH 3.0 - pH 8.5 and the peak at pH 6.0 (87%). The enzyme exhibited high activity with iso-thermal peaks at 40ºC and 80ºC (2024 U/mL) but low stability at most temperatures. Laccase retained 100% and 101.13% activity at 3 mM and 4 mM EDTA, respectively, and 77% activity at 2 mM L-cysteine. Laccase activity was activated by Hg^{2+} (105%), Cu^{2+} (106%), Mg^{2+} (108%) and Mn^{2+} (114.3%). Fe^{2+} (80%), Co^{2+} (90.4%) and Pb^{2+} (91%) showed high inhibition, while Zn^{2+} (62%) and Al^{2+} (63%) showed moderate inhibition. The K_M and V_{max} values of the enzyme were 55.8 µM and 2.10 µMol./min/mL, respectively. The study showed that the purified laccase of *Trametes* sp. G31 has great potentials for applications in industrial and/or biotechnological processes.

Keywords: Enzyme inhibitors; Laccase activity; Metallo-stable laccase; Partial characterization; Total soluble protein; *Trametes* sp.G31

Introduction

Laccase (benzendiol: oxygen oxidoreductase E.C.1.10.3.2) is a copper containing enzyme, part of the group called blue oxidases (Brazkova *et al*., 2016). It is distributed in higher plants, many fungi, some bacteria and insects in nature. They catalyze a vast spectrum of substrates including poly-phenols, substituted phenols, diamines and some inorganic compounds (Viswanath *et al*., 2014). The largest numbers of laccases reported are from fungi belonging to basidiomycetes, ascomycetes and also from deuteromycetes (Sahay, 2021). And the *Trametes* species are the most studied basidiomycete characterized for laccase production (Sahay, 2021). Other species of *Pleurotus, Podospora, Rhizonia, Neurospora, Aspergillus, Phlebia, Botrytis, Cerrena and Myceliophthora* have also been reported.

Laccases from different origins vary considerably in substrate specificity, optimum pH, temperature, molecular weight, metal tolerance and resistance to inhibitors. Laccase are easier to manipulate than both lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) due to their heterogenicity (Budda *et al*., 2012). Therefore, they are more amenable to various processes involving bioremediation, such as degrading of aromatic pollutants from pulp and paper mill effluent (Kumar and Chandra, 2020; Khan *et al*., 2021), olive mill wastewater, polycyclic aromatic

hydrocarbons (Wang *et al*., 2018), chlorinated phenols, polychlorinated biphenyls, dioxins, pesticides, explosives and dyes (Ado *et al*., 2019; Rathore *et al*., 2022).

Laccase have also found several applications in the food industry such as stabilizing agent in beverage processing, ascorbic acid determination, sugar beet pectin gelatinization, and enhancement of bread quality in baking and as biosensors (Agrawal *et al*., 2018; Zrinski *et al*., 2020; Backes *et al*., 2021). Laccase is also useful in the production of environmentally friendly wood-based panels with high hardness and formaldehyde release (Zhou *et al*., 2017). In paper-making industry, laccase is used for the bio-bleaching of paper pulp through laccase-mediator systems or a combination of laccase and other enzymes (Zhou *et al*., 2017). The use of laccase in the textile industry as alternative bio-bleaching system to chemical bleaching is growing very fast. In addition to decolourizing of textile effluents (Sondhi *et al*., 2018), laccases are recently used in the synthesis of industrial dyes (Atav *et al*., 2021).

Although, the desire for relevant laccases has increased due to array of applications in industry and biotechnology; the high costs of production, low enzyme yield, low enzyme activity and stability have limited large-scale applications (Asgher *et al*., 2012; Pang *et al*., 2016). The thermo-stability of a laccase varies considerably with the source of organism. Generally, laccases are stable at 30 - 50°C and rapidly lose activity at temperatures above 60°C (El Monssef *et al*., 2016). The majority of fungal laccases operate in the range of 30 - 55°C, and their optimum pH range is limited to mildly acidic conditions. Reportedly, the optimum temperature for laccase produced by *Pleurotus ostreatus* was approximately 30^{o}C (Elsayed *et al*., 2012), whereas, for *Pleurotus pulmonarius* and *Pleurotus florida* the optimum temperature was at 50^{o}C (Afreen *et al*., 2017). The optimum pH of the purified laccase produced by *Spirulina platensis* was pH 3.0 with ABTS substrate (Afreen *et al*., 2017). Similar pH was reported for laccase produced by *Pleurotus* sp., *Pycnoporus sanguineus* and *Ganoderma lucidum* (Afreen *et al*., 2017).

Studies have shown that the presence of some exogenous ions has a noticeable impact on laccase activity, and greatly affects the efficiency of laccase in practical applications (Zhou *et al*., 2017). Some metals (Mg^{2+}, Cr^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, and Co^{2+}), especially Cu^{2+}, caused strong stimulation for the enzyme, while Fe^{2+} and Hg^{2+} ions caused a reduction in laccase activity even in trace amounts (Zhou *et al*., 2017; Abdelgalil *et al*., 2020). Studies on the effects of various enzyme inhibitors on laccase activity showed sodium azide (98.23%) as the strongest laccase inhibitor, followed by L-cysteine (94.61%), Thioglycolic acid (82.41%), Thiourea (60.23%), and EDTA (30.64%) in descending order (Afreen *et al*., 2017).

Based on the limiting factors such as varying pH activity and stability, low thermotolerance, there is therefore the need to search for more active and stable laccases with required pH and temperature; capable of resisting enzyme inhibitors and metallic ions for large-scale applications in industry and biotechnology. In our present study, *Trametes* sp. G31 previously isolated from Gboko plank market, Gboko was utilized. The fungal strain was used to produce laccase on saw-dust supplemented with a lignin modified medium. The laccase was partially purified and characterized. Laccase activity and stability in the presence of pH, temperature, enzyme inhibitors and metal ions were studied in this present investigation.

Materials and Methods

Isolation and Identification of Fungus

The fungus used in this study was isolated from a saw-dust dump site in Gboko plank market, Gboko, Benue State, Nigeria (Ado *et al*., 2019).

Collection and Processing of Substrate

Wood samples of *Terminalia superba* Engl. & Diels were collected from Gboko plank market, located at Adekaa in Gboko Town, Benue State, Nigeria. The samples were passed through an

electric sliding-table saw machine to obtain wood blocks, oven dried (to constant weight at 80°C) and processed to saw-dust as earlier described (Ado *et al*., 2019).

Media and Culture Conditions

Lignin Modifying Medium (LMM) containing the following composition (g/L): glucose 10 g, Ammonium tartrate 2 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 0.5 g, KCl 0.5 g, Yeast extract 1 g, Soy tone 5 g, $CuSO_4.5H_2O$ (150 µm), EDTA 0.5 g, $FeSO_4$ 0.2 g, $ZnSO_4$ 0.0 1 g, $MnCl_2.4H_2O$ 0.00 3 g, H_3BO_4 0.03 g, $CoCl_2.6H_2O$ 0.02 g, $CuCl_2.2H_2O$ 0.001 g, $Na_2MoO_4.2H_2O$ 0.003 g (Poojary *et al*., 2012); used to moisten the saw-dust sample was adjusted to pH 3.0 - 8.0. Saw-dust was adjusted to approximately 70% moisture content following the wet basis method (Markson *et al*., 2012; Osibe and Chiejina, 2015). Ten milliltres of the medium was added to100 g of the saw-dust in 250 mL Erlenmeyer flask and sterilized by autoclaving at 121°C for 20 minutes. One percent (w/v) aqueous glucose solution was separately autoclaved at 110°C (10 psi) for 10 minutes and 2 mL aseptically added to the fermenting flask. Duplicate flasks were inoculated with two 5 mm agar plugs of actively growing mycelia from a 5-day old fungi culture on PDA and incubated at 27°C ± 2°C for 6 – 34 days as previously discussed by Ado *et al*. (2019).

Extraction of Extracellular Enzymes

Extracellular enzymes were extracted by adding 100 mL 0.1 M citrate-phosphate buffer (pH 5.0) into the fermenting flask. The mixture was stirred with a glass rod for 30 minutes and filtered with cheese-cloth to remove saw-dust and fungal mycelia. The crude filtrate was then filtered with 90 mm Whatman No. 1 filter paper to obtain a clear filtrate which was refrigerated at 4°C (Gomes *et al*., 2009).

Assay of Laccase Activity

Laccase activity was determined at 420 nm with Spectrophotometer using 2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid (ABTS). The reaction mixture consisted of 600 µL sodium acetate buffer (0.1 M, pH 5.0 at 27°C), 300 µL ABTS (5 mM), 300 µL crude laccase and 1400 µL distilled water. The reaction was incubated for 2 minutes at 30°C and initiated by adding 300 µL H_2O_2 and absorbance measured after one minute (Urairuj *et al*., 2003). One Unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1µmol of ABTS (ε=36,000 M^{-1} cm^{-1}) per minute under the specified assay condition (Masalu, 2016).

Protein Determination

Protein concentration was quantified with Folin Ciocalteu's phenol reagent following standard protocol and known concentrations of egg albumin used to extrapolate the standard curve (Sivakumar *et al*., 2010).

Purification of Laccase

The crude laccase was subjected to ammonium sulphate precipitation at 80% (w/v) in an ice bath. The saturated solution was maintained overnight at 4°C and precipitate was allowed to sediment by repeating the above procedure. Pellets were collected and reconstituted while concentrated samples with maximum laccase activity were dialyzed overnight using dialysis tubing (MWCO 12 - 14 kDa) as previously described (Aslam and Asgher, 2011; Ado *et al*., 2018b).

Characterization of Laccase

The effect of pH on laccase activity was determined at different pH values. Laccase activity at pH 3.0 was assayed in 20 mM Succinate buffer, pH 4.0 - 5.0 in 50 mM malonate buffer, pH 6.0 - 7.0 in 100 mM phosphate buffer, and pH 8.5 in 100 mM sodium phosphate buffer (Irshad and Asgher, 2011) following the standard protocol described under assay of laccase activity. Laccase stability was determined by incubating the enzyme (1:1) in 0.1 M pH buffers; pH 3.0 - 5.0 (sodium acetate), pH 5.0 - 7.0 (citrate-phosphate) and pH 7.0 - 8.5 (tris-HCl) at 25°C for 24 hours. A 300 µL aliquot was used to determine the remaining activity at optimum pH and temperature (Gomes *et al*., 2009; Budda *et al*., 2012). The effect of temperature on laccase activity was carried out at 30°C - 90°C for 15 minutes at optimum pH following the standard protocol (Irshad and Asgher, 2011). Laccase stability was evaluated

at 20°C - 90°C for 1 hour using optimum pH. A 300 µL aliquot enzyme was withdrawn and placed on ice before assaying for remaining activity (Gomes *et al*., 2009; Budda *et al*., 2012).

The effect of metal ions on laccase activity was determined by incubating the reaction mixture of 300 µL enzyme, 800 µL of 0.1 M sodium acetate buffer containing ABTS (0.18 mM, pH 4.5) and 300 µL metal ion solution at 30°C for 30 minutes. The metal ions Cu^{2+}, Mg^{2+}, Pb^{2+}, Hg^{2+}, Mn^{2+}, Al^{3+},Zn^{2+}, Fe^{2+} and K^{+} in their chloride forms were used at the concentration of 1 – 5 mM. After incubation, the remaining enzyme activity was assayed. A heat-denatured enzyme was used as control (Saito *et al*., 2003; Sadhasivam *et al*., 2008; Stoilova *et al*., 2010). The effect of EDTA and L-cysteine on laccase activity was determined by incubating 1.4 mL reaction mixture comprising 800 µL of 0.1 M sodium acetate buffer containing ABTS (0.18 mM, pH4.5), 300 µL of enzyme and 300 µL of inhibitor at various concentrations 1 – 5 mM. Incubation was at 30°C for 30 minutes and the absorbance measured at 436 nm using Spectrophotometer. A control test was conducted in the absence of the inhibitor (Sadhasivam *et al*., 2008; Irshad and Asgher, 2011).

The Michaelis-Menten kinetic parameters (K_M, V_{max}) were determined by measuring laccase activity at varying concentrations of ABTS from 0.1 mM - 0.5 mM. The parameters were obtained by curve fitting the reciprocal plot of reaction rate (V) versus substrate concentration (S) using Linweaver-Burk plot (Farnet *et al*., 2004).

Statistical Analysis

Results obtained from this study were subjected to analysis of variance using one way ANOVA and differences between means of test samples were separated by Duncan Multiple Range Test (Ducan, 1955).

Results and Discussion

The effect of fermentation period on laccase production and total soluble protein is presented in Fig. 1. In the study, laccase production increased gradually from day 6 (1721 U/mL) to its peak on day 14 (2113 U/mL). Thereafter, production of laccase generally remained fairly stable as fermentation progressed. However, TSP production declined continuously throughout the fermentation period from 2.52 mg/mL (day 6) to 1.94 mg/mL and 1.4 mg/mL on day 18 and day 34, respectively. Ergun and Urek (2017) reported maximum laccase activity after the activity of protease declined on day 7 during the SSF of banana peels by *Pleurotus ostreatus*. Therefore, it is possible that the continuous decline in TSP production was probably due to the activity of proteases that were secreted by *Trametes* sp. G31. Several researchers have reported optimum production of crude laccases at different periods of incubation. Some studies obtained maximum production of laccase on day 7 and day 10 using *Lentinus edodes* and *Ganoderma sp*. respectively; while, another work reported maximum production of laccase on day 11 using saw-dust from rubber wood (Elisashvili *et al*., 2008; Sivakumar *et al*., 2010*;* Ding *et al*., 2012). However, another study recorded maximum production of laccase by *Ganoderma lucidum* on day 16 which is close to this finding (Aftab and Ahmad, 2015). In our earlier study, maximum production of TSP and laccase was obtained on day 14 and day 18 respectively using *Trametes* sp. isolate B7 grown on saw-dust of *Terminalia superba* (Ado *et al*., 2018a). It has been established that depending on the fungal strain and culture medium the peaks of laccase production varies with days of incubation (Elisashvili and Kachlishvilli, 2009). Thus, the differences in optimum incubation periods could be due to differences in fungi species and their abilities to metabolize different lignocellulosic substrates (Elsayet *et al*., 2012).

Fig. 2 presents the effect of pH on laccase and TSP production by *Trametes* isolate G31. The optimum pH for laccase production ranged from pH 5.0 (2356 U/mL) - pH 7.0 (2369 U/mL) and

coincided with the lowest TSP productions of 1.4 mg/mL and 1.70 mg/mL respectively. Similarly, the highest TSP at pH 3.0 (2.50 mg/mL) coincided with lowest laccase production (342 U/mL). It has been established that TSP (enzymic and non enzymic proteins) is produced in higher yield at a pH near the optimum for microbial physiology (Chanakya *et al*., 2010). Thus, the pH of culture medium affects growth and secretion of TSP. However, its optimum is specie and strain-specific coupled with the type of lignocellulosics used (Elisashvili and Kachlishvili, 2009; Chanakya *et al*., 2010). Several works have shown that production of fungal enzymes by most strains of fungi is best at pH 4.0 - 6.0 (Sivakumar *et al*., 2010; Singh and Abraham, 2013). Other authors have reported maximum production of laccase by several fungi species including *T. versicolor* within the range of pH 3.5 - 7.0 which corroborates our work.

Fig. 3 presents the activity and stability of the partially purified laccase. The highest activity of laccase was obtained at pH 3.0 - 5.0, laccase stability was high at pH 3.0 - pH 8.5 and the peak was obtained at pH 6.0 (87%). Contrary to this study, laccase of *Alcaligenes faecalis* showed high stability at pH 4.0 – 5.0 but was unstable at pH 6.0 (49.1%) after 5-hour incubation (Abdelgalil *et al*., 2020). Thus, the activity and stability of laccase is very crucial in its choice and application in different areas of industry and biotechnology. Many reports show that the optimum pH for laccase activity depend on the substrate used for assay. Reportedly, many laccases have optimal catalytic pH values in the acidic range using the substrate ABTS (Ding *et al*., 2012). Partial characterization of purified laccases from *Cladosporium cladosporioides* and *Trametes* sp. isolate B7 showed wide pH optima of 3.0 - 6.0 using ABTS (Aslam *et al*., 2012; Ado *et al*., 2018b). In another study, the characterization of extracellular laccases from *Trametes versicolor*, *Fomes annosus*, *Pluerotus ostreatus*, *Rhizoctonia praticola and Botrytis cinerea* observed that optimum activity varied between pH 3.0 - 5.0 using ABTS which is consistent with our work (Rasera *et al*., 2009). The high stability of *Trametes* sp. G31 partially purified laccase obtained at a wide pH range (3.0 - 8.5) makes it a potential candidate for many processes that require acidic to slightly alkaline medium in industry and biotechnology.

Fig. 4 shows two iso-thermal peaks of laccase activities (2024 U/mL) each at 40ºC and 80ºC. Maximum laccase activity from *T. versicolor* was achieved at 40°C (Hossain and Anantharaman, 2006) which falls within the lower range observed in this study. However, the enzyme exhibited low stability at most temperatures with 12.4% (40ºC) and 7.31% (80ºC) for 1 hour. To overcome the draw-back, the laccase of *Trametes* sp. isolate G31 could be immobilized to increase its thermal stability and enhance wider applications in research and industry. In other studies, laccase of *K. pneumonia* were stable up to 70°C (Gaur *et al*., 2018), while laccase of *Cladosporium cladosporioides* were stable from 40 - 70°C but with optimum temperature at 40°C which supports our findings (Aslam *et al*., 2012).

The effect of EDTA and L-cysteine on inhibition of laccase activity is presented in Fig. 5. In general, the metallo-enzyme inhibitor EDTA exhibited very weak inhibitory effects on laccase. At 1 mM, 2 mM and 5 mM concentrations, the enzyme retained 94%, 89% and 86% activities respectively; while at 3 mM and 4 mM concentrations laccase activities were 100% and 101.13% respectively. However, L-cysteine exhibited stronger inhibition than EDTA (Afreen *et al*., 2017), with lower laccase activities of 62% and 61.1% at 1 mM and 5 mM, and higher laccase activity of 77% at 2 mM. Studies have shown that EDTA is an inhibitor of metallo-enzymes including laccase due to its property of forming inactive complexes with inorganic prosthetic groups/co-factors of the enzyme (Sadhasivam *et al*., 2008). Like-wise, L-cysteine is a stronger inhibitor of laccase activity than EDTA which agreed with this study (Aslam and Asgher, 2011; Ado *et al*., 2018a). The ability to with stand the inhibitory effect of EDTA and moderate effect of L-cysteine made the enzyme a great candidate for industrial and biotechnological processes including

bioremediation where resistance to inhibitors is critical.

Fig. 6 presents the effect of different metallic ions on laccase activity. Two out of the nine metal ions investigated; namely, Zn^{2+} and Al^{3+} showed mild inhibitory effects. The enzyme retained 62% (Zn^{2+}) and 63% (Al^{3+}) activity while Fe^{2+}, Co^{2+} and Pb^{2+} retained 80%, 90.4% and 91% laccase activity respectively. It was established that in the presence of various salts, metallic ions substantially affect enzyme activity (Stoilova *et al*., 2010). However, Cu^{2+}, $Hg^{2+}$and Mg^{2+} activated laccase activity with 106%, 105% and 108% respectively. Mn^{2+} ions activated the highest laccase activity by 114.3%, 113.4% and 114.2% at 3 mM, 4 mM and 5 mM respectively. In another study, Mn^{2+}, and Mg^{2+} had high stabilizing effects on laccase from *T. versicolor* while Zn^{2+} and Cu^{2+} had destabilizing effects and in extreme cases complete loss of enzyme activity was recorded in the presence of Cu^{2+} and Fe^{2+} (Stoilova *et al*., 2010). However, in our study Mn^{2+}, Mg^{2+} along Cu^{2+} activated laccase activity while Fe^{2+} exerted weak inhibition of the enzyme. Higher inhibitory rates of 64% and 55% for Zn^{2+} and K^{+}, respectively was reported for purified laccase of *Lentinus edodes* (Sadhasivam *et al*., 2008). These variations is because the effect of metal ions on laccase activity is highly dependent on its source and the type of metals used, which have a great influence on the catalytic activity of the enzyme (Sadhasivam *et al*., 2008).

Fig. 7 presents the Lineweaver-Burk reciprocal plot of the purified laccase of *Trametes* sp. isolate G31. The purified laccase had K_M and V_{max} values of 55.8 µM and 2.10 µMol./min/mL, respectively. This is close to the K_M (33 µM) and V_{max} (1.91 µMol./min/mL) values obtained from the laccase produced by *Trametes* sp. B7 in our previous study. Another study reported higher K_M (180 µM) and Vmax (3.95 µmol/min/mg) for purified laccase of *T. harzianum* using ABTS (Sadhasivam *et al*., 2008). This implied that the partially purified laccase of *Trametes sp.* isolate G31 had greater substrate affinity than that of *T. harzianum*. This is because the rate of substrate-enzyme reaction depends on its K_M, and enzymes with low K_M values have higher affinity for the substrate (Irshad and Asgher, 2011).

Conclusion

The laccase from *Trametes* sp. G31 showed considerable activity at pH 5 - 7 after 14 days of SSF fermentation using saw-dust as the sole source of carbon. The enzyme was optimally active at pH 3.0 - 5.0, with wide range stability with in pH 3.5 - 8.5. The enzyme displayed fantastic degree of stability to varying metal ions tested and lower inhibition on exposure to inhibitors. Therefore, the properties of the purified laccase made it a great tool for applications in industrial and or biotechnological processes. However, the low thermal stability of the enzyme requires immobilization for enhanced stability. Therefore, future work on immobilization of the laccase for industrial applications is imperative.

Declaration of conflicting interest

The authors declared no potential conflicts of interest.

References

Abdelgalil SA, Attia AR, Reyed RM & Soliman NA 2020. Partial purification and biochemical characterization of a new highly acidic NYSO laccase from *Alcaligenes faecalis*.*Journal of Genetic Engineering and Biotechnology*, 18:79 https://doi.org/10.1186/s 43141-020-00088-w.

Ado BV, Amande TJ, Ebah EE & Mabitine DM 2018b. Screening, production and partial characterization of a thermostable laccase from *Trametes sp*. isolate B7 with biotechnological potentials. *Biotechnology Journal International*, 22(4): 1 - 16.

Ado BV, Onilude AA & Amande TJ 2018a. Utilization of *Terminalia superba* saw-dust as substrate for laccase production by *Trametes* sp. isolate B7 under solid state fermentation. *Microbiology Research Journal International*, 26(3): 1 - 12.

Ado BV, Onilude AA, Oluma HOA & Mabitine DM 2019. Production of fungal laccase under solid state bioprocessing of agroindustrial waste and its application in decolourization of synthetic dyes. *Journal of Advances in Biology & Biotechnology*, 21(4): 1-17.

Afreen S, Shamsi TN, Baig MA, Ahmad N, Fatima S, Qureshi MI, Hassan MI & Fatma T 2017. A novel multicopper oxidase (laccase) from cyanobacteria: Purification, characterization with potential in the decolorization of anthraquinonic dye. *PLOS ONE*, https://doi.org/10.1371/journal.pone.0175144.

Aftab ZH & Ahmad S 2015. *Ganoderma lucidum*: A case study for laccase biosynthesis. *Pakistan Journal of Phytopathology*, 27(01): 95 - 103.

Agrawal K, Chaturvedi V, & Verma P 2018.Fungal laccase discovered but yet undiscovered. *Bioresources and Bioprocessing*, *5*(1): 1-12.

Asgher M, Kamal S & Iqbal HMN 2012. Improvement of catalytic efficiency, thermo-stability and dye decolorization capability of *Pleurotus ostreatus* IBL -02 laccase by Hydrophobic Sol Gel Entrapment. *Chemistry Central Journal*, 6(1): 110. DOI:10.1186 /1752-153X-6-110.

Aslam MS, Aishy A, Samra ZQ, Gull I & Athar MA 2012. Identification, purification and characterization of a novel extracellular laccase from *Cladosporium cladosporioides*. *Biotechnology & Biotechnological Equipment*, 26(6): 3345 - 3350.

Aslam S & Asgher M 2011. Partial purification and characterization of ligninolytic enzymes produced by *Pleurotus ostreatus* during solid state fermentation. *African Journal of Biotechnology*, 10(77): 17875 - 17883.

Atav R, Bugdaycı B, & Yakın İ 2021. Laccase-catalyzed simultaneous dye synthesis and cotton dyeing by using plant extracts as dye precursor. *The Journal of the Textile Institute*, 1-9.

Backes E, Kato CG, Correa RCG, Moreira RDFPM, Peralta RA, Barros L, & Peralta RM 2021. Laccases in food processing: Current status, bottlenecks and perspectives. *Trends in Food Science & Technology*, 115: 445 - 460.

Brazkova M, Mercati A, Hristova I, Lante A & Krastanov A 2016. Isolation, purification and characterization of laccase from the white rot fungus *Trametes versicolor*. *Scientific Works of Food Technology*, 63(1): 155 - 162

Budda W, Sarnthima R, Khammuang S, Milintawisamai N & Naknil S 2012. Ligninolytic enzymes of *Lentinus polychrous* grown on solid substrates and its application in black liquor treatment. *Journal of Biological Sciences*, 12(1): 25 - 33.

Chanakya P, Manipati S & Somalanka SR 2010. Process optimization of L-glutaminase production by *Trichoderma koningii* under solid state fermentation. *Journal of Applied Biology and Pharmaceutical Techonolgy*, 3: 1168 - 1174.

Ding Z, Peng L, Chen Y, Zhang L, Gu Z, Shi G & Zhang K 2012. Production and characterization of thermo-stable laccase from the mushroom, *Ganoderma lucidum*, using submerged fermentation. *African Journal of Microbiology Research*, 6(6): 147 - 1157.

Ducan DB 1955. Multiple range and multiple F tests. *Biometrics*, 11(1): 1 - 42.

El Monssef RAA, Hassan EA & Ramadan EM 2016. Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment. *Annals of Agricultural Science*, 62(1): 145 - 154.

Elisashvili V & Kachlishvili E 2009. Physiological regulation of laccase and manganese peroxidase production by white-rot *Basidiomycetes*. *Journal of Biotechnology*, 144: 37 - 42.

Elisashvili V, Penninckx M, Kachlishvili E, Tsiklauri N, Metreveli E, Kharziani T & Kvesitadze G 2008. *Lentinus edodes* and *Pleurotus* species lignocellulolytic enzymes activity in submerged and solid-state fermentation of lignocellulosic wastes of different composition. *Bioresources Technology*, 99: 457 - 462.

Elsayed MA, Hassan MM, Elshafei AM, Haroun BM & Othman AM 2012. Optimization of cultural and nutritional parameters for the production of laccase by *Pleurotus ostreatus* ARC280. *British Biotechnology Journal*, 2(3): 115 - 132.

Ergun SO & Urek RO 2017. Production of ligninolytic enzymes by solid state fermentation using *Pleurotus ostreatus*. *Annals of Agrarian Science*, 15(2017): 273 – 277.

Farnet AM, Criquet S, Cigna M, Gil G & Ferre E 2004. Purification of a laccase from *Marasmius quercophilus* induced with ferulic acid: Reactivity towards natural and xenobiotic aromatic compounds. *Enzyme Microbiology and Technology*, 34: 549 – 554

Gaur N, Narasimhulua K & Pydisetty Y 2018. Biochemical and kinetic characterization of laccase and manganese peroxidase from novel *Klebsiella pneumoniae* strains and their application in bioethanol production. *The Royal Society of Chemistry Advances*, 8: 15044 - 15055.

Gomes E, Aguiar AP, Carvalho CC, Bonfa MRB, Silva R & Boscolo M 2009. Ligninase production by basidiomycetes strains on lignocellulosic agricultural residues and their application in the

decolourization of synthetics dyes. *Brazilian Journal of Microbiology*, 40: 31 - 39.

Hossain SM & Anantharaman N 2006. Activity enhancement of ligninolytic enzymes of *Trametes versicolor* with bagasse powder. *African Journal of Biotechnology*, 5(1): 189 - 194.

Irshad M & Asgher M 2011. Production and optimization of ligninolytic enzyme by white rot fungus Schizophyllum commune IBL-06 in solid state fermentation medium banana stalk. *African Journal of Biotechnology*, 10: 18234 - 18242.

Khan SS, Zarin A, Ahmed S, Hasan F, Belduz AO, Canakci S, Khan S, Badshah M, Farman M & Shah AA 2021. Degradation of lignin by *Bacillus altitudinis* SLT isolated from pulp and paper mill effluent. *Water Science & Technology*, 85(1): 420 – 432.

Kumar A & Chandra R 2020. Ligninolytic enzymes and its mechanism for degradation of lignocellulosic waste in environment. *Heliyon*, 6(2020): eo3170.

Majolagbe ON, Oloke JK, Deka-Boruah HP, Bordoloi AK & Borah M 2012. Extraction of extracellular laccase from wild, mutants and hybrid strains of two white-rot fungus and its applications in decolourization and ligninolysis. *Journal of Science and Technology*, 2: 301 - 317.

Markson AA, Madunagu BE & Enyiko ED 2012. Growth performance of *Pleurotus ostreatus* (Jacq. *et*. Fr.) Kummer on different substrates treated with used automobile engine oil. *International Journal of Recent Scientific Research*, 3(5): 384 -388.

Masalu RJ 2016. Ligninolytic enzymes of the fungus isolated from soil contaminated with cow dung. *Tanzanian Journal of Science*, 42:85 - 93.

Osibe DA & Chiejina NV 2015. Assessment of palm press fibre and saw-dust based substrate formulas for efficient carpophores production of *Lentinus squarrosulus* (Mont.) singer. *Mycobiology*, 43(4): 467 - 474.

Pang S, Wu Y, Zhang X, Li B, Ouyang J & Ding M 2016. Immobilization of laccase via adsorption onto bimodal mesoporous Zr-MOF. *Process Biochem*istry, 51(2): 229 - 239.

Poojary H, Hoskeri A, Kaur A & Mugeraya G 2012. Comparative production of ligninolytic enzymes from novel isolates of basidiomycetes and their potential to degrade textile dyes. *Nature and Science*, 10(10): 90 - 96.

Rasera K, Ferlaa J, Dillona AJP, Riveiros R & Zenib M 2009. Immobilization of laccase from *Pleurotus sajor-caju* in polyamide membranes. *Desalination*. 246: 284 - 288.

Rathore S, Varhney A, Mohan S & Dahiya P 2022. An innovative approach of bioremediation in enzymatic degradation of xenobiotics. *Biotechnology & Genetic Engineering Reviews*, DOI: 10.1080/02648725.2022.2027628.

Sadhasivam S, Savitha S, Swaminathan K & Lin FH 2008. Production, purification and characterization of mid-redox potential laccase from a newly isolated *Trichoderma harzianum* WL1.*Process Biochemistry*, 43: 736 - 742.

Sahay R 2021. Properties of laccase, physiological functions and their industrial applications *International Journal of Recent Scientific Research*. 12(6): 24023 - 24030.

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

Singh N & Abraham J 2013. Isolation of laccase producing fungus from compost soil and partial characterization of laccase. *Advances in Applied Science Research*, 4(5): 91 - 98.

Sivakumar R, Rajendran R, Balakumar C & Tamilvendan M 2010. Isolation, screening and optimization of production medium for thermostable laccase production from *Ganoderma* sp. *International Journal of Engineering Science and Technology*, 2(12): 7133 - 7141.

Sivakumar R, Rajendran R, Balakumar C & Tamilvendan M 2010. Isolation, screening and optimization of production medium for thermostable laccase production from *Ganoderma sp*. *International Journal of Engineering Science and Technology*, 2(12): 7133 - 7141.

Sondhi S, Kaur R, Kaur S, & Kaur PS (2018). Immobilization of laccase-ABTS system for the development of a continuous flow packed bed bioreactor for decolorization of textile effluent. *International Journal of Biological Macromolecules*, 117: 1093-1100.

Stoilova I, Krastanov A & Stanchev V 2010. Properties of crude laccase from *Trametes versicolor* produced by solid-state fermentation. *Advances in Bioscience and Technology*, 1: 208 - 215.

Urairuj C, Khanongnuch C & Lumyoung S 2003. Ligninolytic enzymes from tropical endophytic *Xylariaceae. Fungal Diversity*, 13: 209 - 219.

Viswanath B, Rajesh B, Janardhan A, Kumar AP & Narasimha G 2014. Fungal laccases and their applications in bioremediation. *Enzyme Research*, Available:http://dx.doi.org/10.1155/2014/163242. Accessed 15-8-2016.

Wang X, Sun SY, Ni ZJ, Li ZX & Bao J 2018. Degradation of polycyclic aromatic hydrocarbons in contaminated soil by immobilized laccase. *Journal of Serbian Chemical Society*, 83(5): 549 – 559.

Zhou C, Dong A, Wang Q, Yu Y, Fan X, Cao Y & Li T 2017. Effect of common metal ions and anions on laccase catalysis of guaiacol and lignocellulosic fiber. *BioResources*, 12(3): 5102 - 5117.

Zrinski I, Pungjunun K, Martinez S, Zavasnik J, Stanković D, Kalcher K, & Mehmeti E 2020. Evaluation of phenolic antioxidant capacity in beverages based on laccase immobilized on screen-printed carbon electrode modified with graphene nanoplatelets and gold nanoparticles. *Microchemical Journal*, 152: 104282.

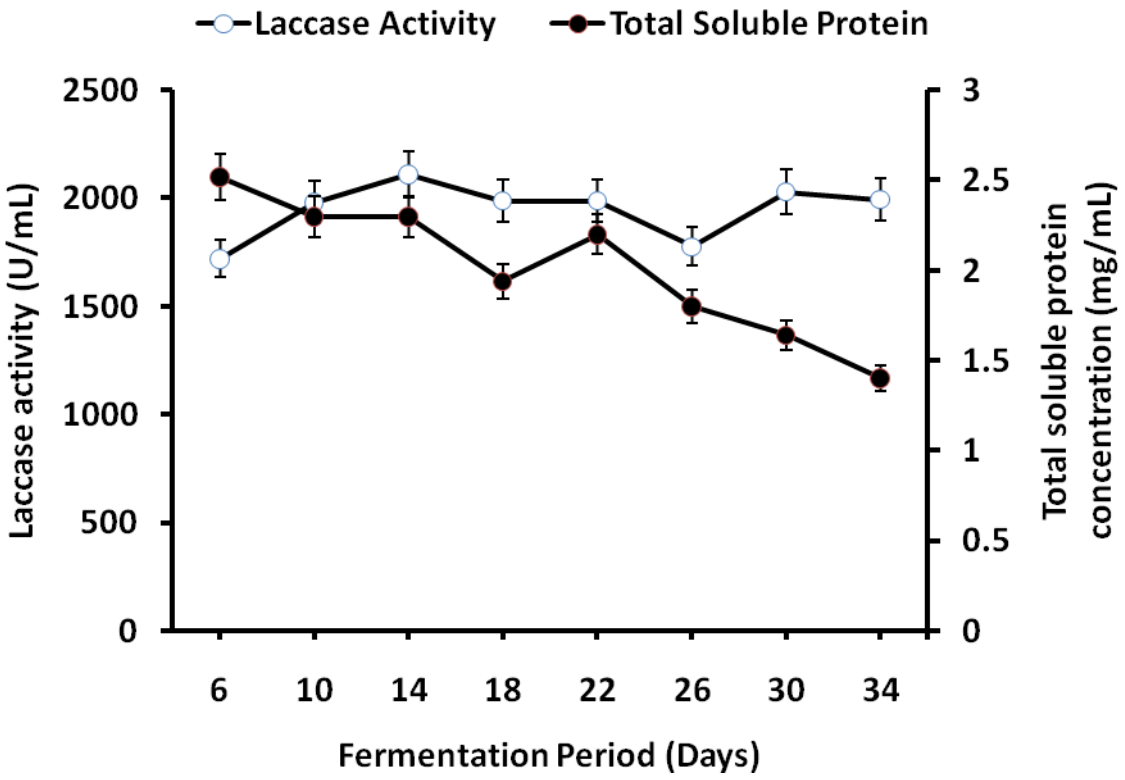

Fig 1: Effect of fermentation period on laccase and total soluble protein production. Bar represent standard error of duplicate determination.

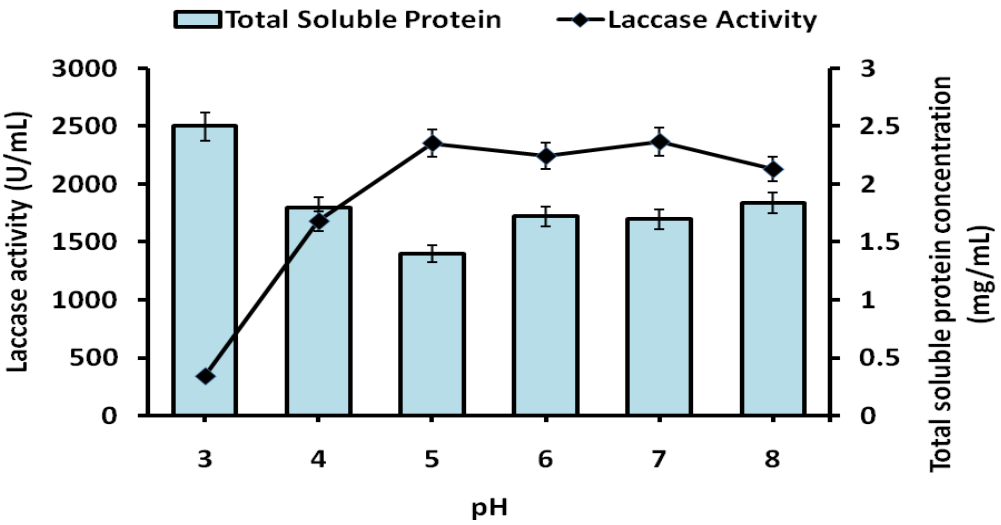

Fig 2: Determination of optimum pH for laccase and TSP production. Bar represent standard error of duplicate determination.

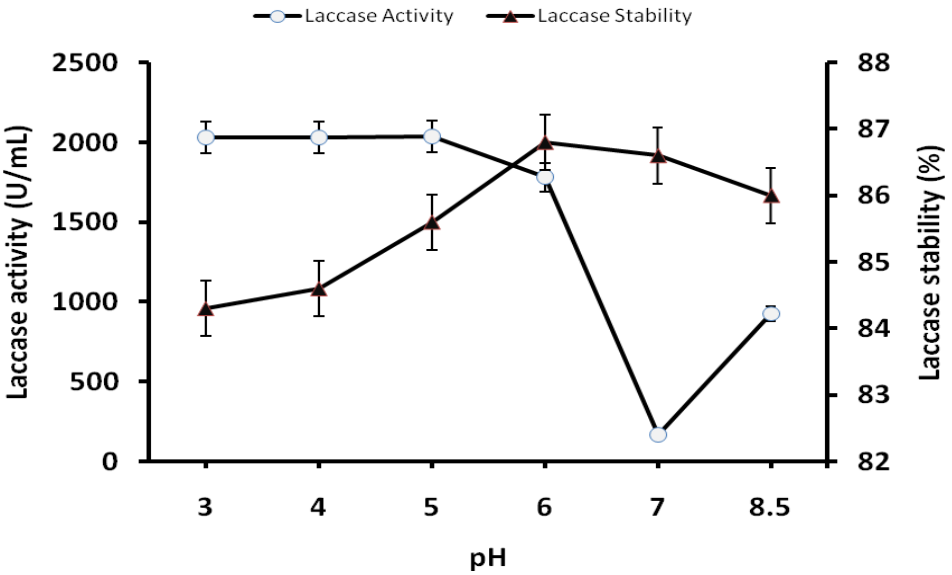

Fig 3: Effect of pH on activity and stability of partially purified laccase. Bar represent standard error of duplicate determination.

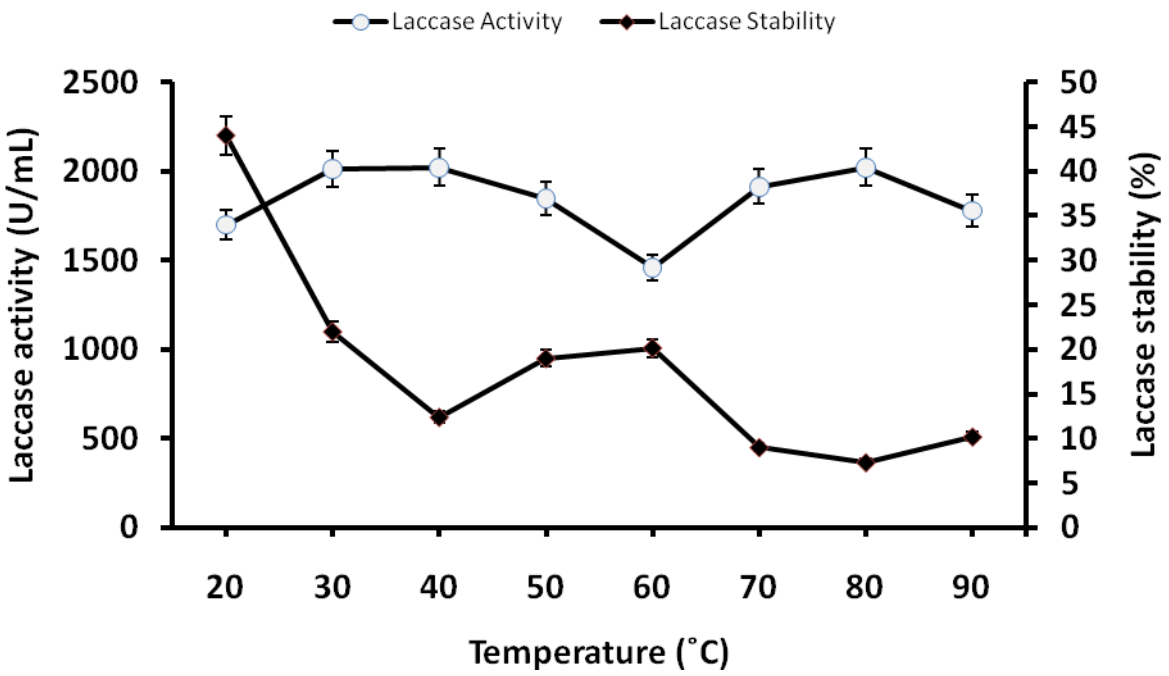

Fig 4: Effect of temperature on activity and stability of partially purified laccase. Bar represent standard error of duplicate determination.

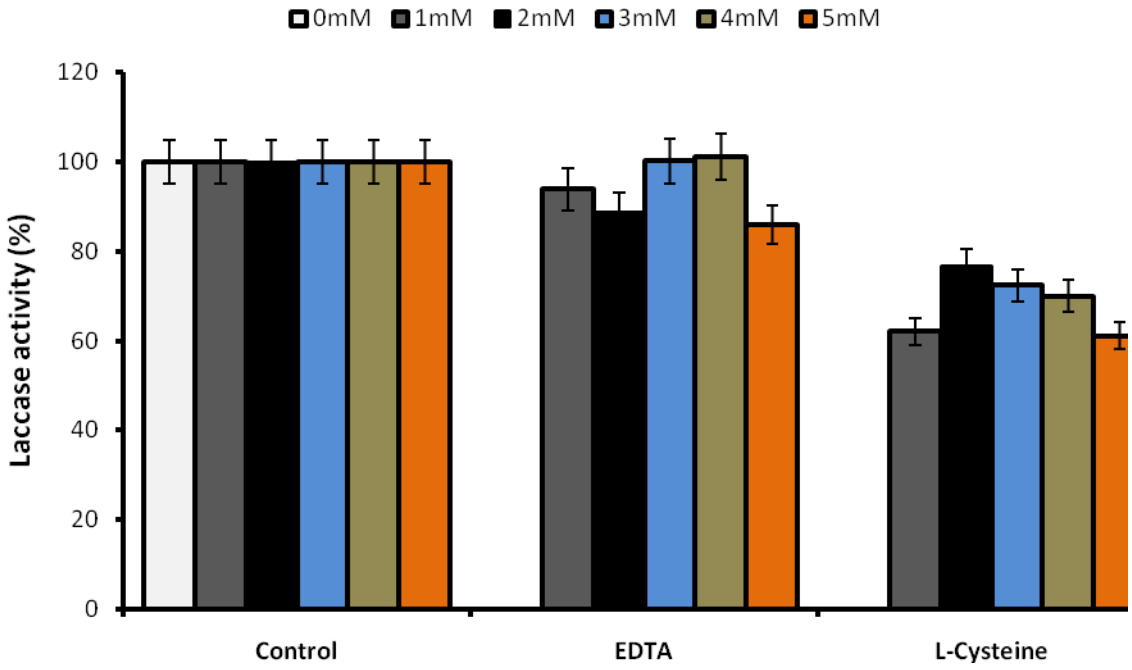

Fig 5: Effect of enzyme inhibitors: EDTA and L-cysteine concentration on the activity of partially purified laccase of *Trametes sp.* G31. Bar represent standard error of duplicate determination.

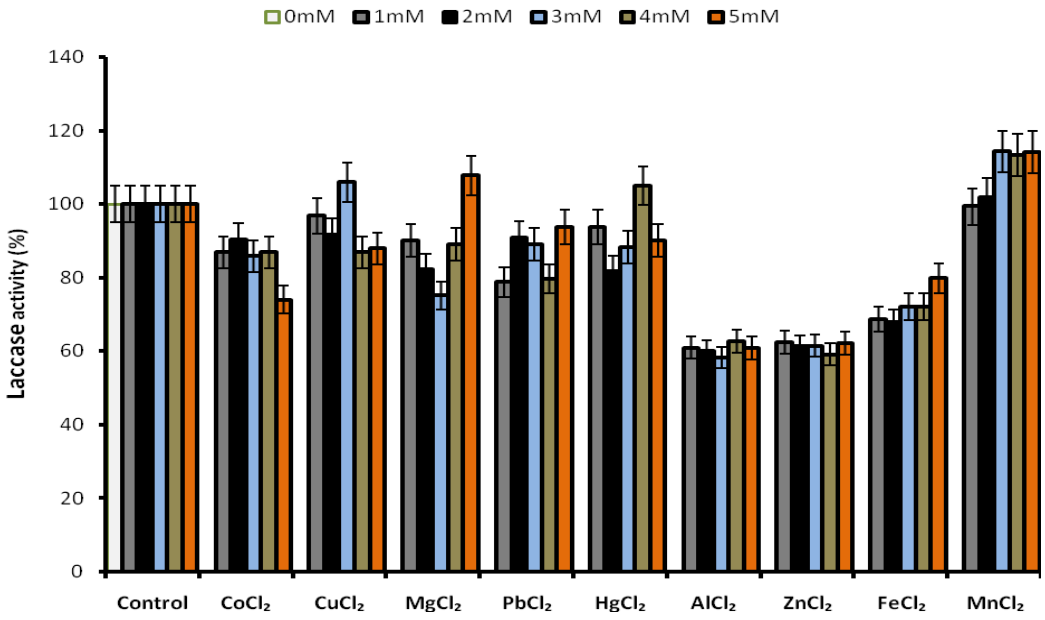

Fig 6: Effect of metal ions on the activity of partially purified laccase of *Trametes sp.* G31. Bar represent standard error of duplicate determination.

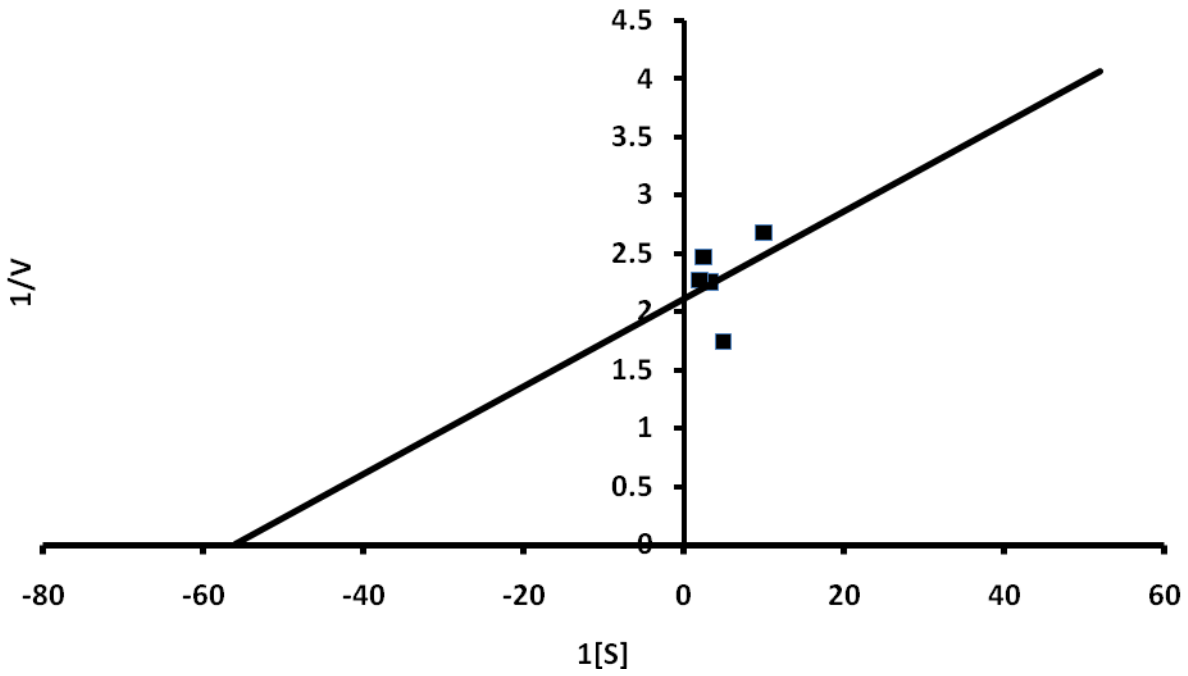

Fig 7: Lineweaver-Burk reciprocal plot: Determination of K_M and V_{max} of purified laccase from *Trametes* sp. 1/V represent (Velocity of reaction) and 1/[S] (Substrate concentration).