Original Research Article

Utilization of *Terminalia superba* Sawdust as Substrate for Laccase Production by *Trametes* sp. isolate B7 under Solid State Fermentation.

ABSTRACT

Laccases catalyze a broad range of substrates due to their low substrate specificity and strong oxidative potentials. Sources include plants, prokaryotes, arthropods and fungi especially *Trametes sp*. In this study laccases were produced by *Trametes sp.* isolate B7 utilizing sawdust as substrate in solid state fermentation. A fraction of the crude enzyme solution was partially purified and characterized. The highest total soluble protein (3.6 mg/mL) and laccase concentration (2356 U/mL) were produced on day 14 and day 18 respectively at pH 5.0. The laccase was 2.3 and 9.0 times purified with 1487 U/mL and 5380 U/mL specific activity for pellets and dialysate respectively. The purified laccase was active in acidic pH 3.0 - pH 6.0 and at 20 ^{o}C - 50 ^{o}C and 80 ^{o}C while stability was highest at pH 6.0 (89% for 24 hr) and 70 ^{o}C (100% for 1 hr). Manganese, Lead, Mercury, Copper and Magnesium ions significantly increased laccase activity whereas Aluminium, Potassium, Iron and Zinc ions decrease activity of the purified enzyme (P = .05). EDTA activated laccase activity at 2 mM (117%) while L-cysteine inhibited enzyme activity at 1 mM - 5mM concentrations. Kinetic studies of the purified laccase showed Km 33 µM and Vmax 1.91 µMol./min/mL with molecular weight of ~36 kDa using N-PAGE. The purified laccase remained active in acidic conditions with high thermostability and resistance to inhibition of most of the metallic ions and EDTA tested. Thus, the enzyme was a versatile tool for biotechnological, industrial and bioremediation processes including polycyclic aromatic hydrocarbons, pesticides and dye waste waters among other xenobiotics.

Keywords: Laccase; Trametes sp. isolate B7; *solid state fermentation; sawdust; laccase activity and stability.*

1. INTRODUCTION

Lignocellulose is the major structural component of both woody and non-woody plants and represents a major source of renewable organic matter [1]. Large amounts of lignocellulosic "waste" such as sawdust are generated through many practices including timber mills which constitute environmental pollution. Production of fungal laccases using sawdust as substrate in Solid State Fermentation (SSF) is an attractive alternative process due to its lower capital investment and lower operating cost [2].

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are multicopper blue oxidases widely distributed in higher plants, in some insects and in a few bacteria [3]. However the best known laccases are of fungal origin. Laccase production occurs in various fungi over a wide range of taxa. Among them, basidiomycetes are efficient laccase producers, especially white rot fungi [4].Well known laccase producers include *Trametes versicolor, Chaetomium thermophilum* and *Pleurotus eryngii* [5].

Laccases are either monomeric or multimeric glycoproteins and their heterogeneity is dependent on variations in carbohydrate content or differences in copper content [6]. They catalyze a broad range of substrates including poly-phenols, substituted phenols, diamines and other aromatic compounds [3]. Their range of action can extend to other substrates by the addition of small molecules, which act as mediators, to the reaction system [7]. Laccases from different organisms show considerable diversity in substrate specificity, molecular weight, pH optimum and other properties.

Interest in laccases has increased considerably because of the variety of biotechnological applications. Due to their low substrate specificity and strong oxidative abilities, laccases have a number of industrial applications including biopulping, prevention of wine decolouration, detoxification

of environmental pollutants, textile dye bleaching, enzymatic conversion of chemical intermediates and the production of valuable compounds from lignin [8, 9, 10].

The ideal laccases for industrial use would exhibit activity and stability at high temperature and wide pH conditions [11, 12]. Thus, fungal strains with high laccase activity and substrate affinity that exhibit such stabilities have a potential for biotechnological applications [10]. The production of laccase by basidiomycetes using submerged fermentation has been reported extensively, even though these organisms grow in nature under solid-state conditions [13]. However, reports on laccase production in solid-state fermentation are scanty. Owing to the diversity of applications for laccases, it's important to identify new sources of the enzyme with novel properties suitable for industrial use.

The objective of this study was to produce novel laccases from *Trametes sp.* isolate B7 with high enzyme activity and stability over wide pH range, high temperatures, and resistant to inhibitory effects of metallic ions and EDTA; and capable of industrial and biotechnological applications using cheap substrate such as sawdust in SSF.

2. MATERIALS AND METHODS

2.1 Substrate Collection and Preparation

Wood samples of *Terminalia superba* Engl. and Diels were collected from Gboko plank market, Benue State, North-Central Nigeria. The samples were passed through an electric sliding-table saw machine to obtain wood blocks which were oven dried to constant weight at 80 ^{o}C. The blocks were directly fed into a motorized rotary machine and crushed into sawdust particles. The sawdust was then passed through a 2 mm wire mesh of metallic sieve to obtain particles of even sizes so that fungal growth would not differ due to differences in oxygen diffusion, nutrient absorption and assimilation by mycelia [14]. The substrate was then dispensed into and sealed in plastic bags and stored in the laboratory.

2.2 Isolation and Identification of Fungal strain

The fungus used in this study was isolated from a decaying wood in Benue Polytechnic Campus, Ugbokolo, Benue State, Nigeria. Pieces of sample were placed on fully sterile Potato Dextrose Agar (PDA) plates and incubated at 27 ^{o}C ± 2 ^{o}C for 7 days. Pure cultures were obtained by sub-culturing onto fresh sterile PDA plates and placed on PDA slants which were refrigerated at 4 ^{o}C. Five-day old fungal cultures on PDA plates were observed for both cultural and morphological characteristics [15]. The fungal isolate was identified using molecular and phylogenetic characterization as earlier described [16]. The sequence was deposited in GenBank under the accession number MK024175.

2.3 Media and Culture Conditions

The Lignin Modifying Medium (LMM) used to moisten the sawdust sample was adjusted to pH 3.0 - 8.0 and had the following composition (gL^{-1}) 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 1g, $MnCl_2.4H_2O$ 0.00 3g, 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 [17]. Ten millilitres of the medium was added to 100 g of the sawdust with approximately 70% moisture content in 250 mL Erlenmeyer flask and sterilized by autoclaving at 121 ^{o}C for 20 minutes. One percent (w/v) aqueous glucose solution was separately autoclaved at 110 ^{o}C (10 psi) for 10 minutes and 2 mL aseptically added to the fermenting flask. Flasks were allowed to cool then aseptically inoculated with two 5 mm agar plugs of actively growing mycelia from a 5-day old fungi culture on PDA .Flasks were prepared in duplicate and incubated at 27 ^{o}C ± 2 ^{o}C for 6,10,14,18,22,26,30 and 34 days [18].

2.4 Extraction of Extracellular Enzymes

Extracellular enzymes were extracted by addition of 100 mL of 0.1M 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 sawdust 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 ^{o}C [19].

2.4.1 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 ^{o}C), 300 µL ABTS (5 mM), 300 µL culture supernatant and 1400 µL distilled water. The reaction was incubated for 2 minutes at 30 ^{o}C and initiated by adding 300 µL H_2O_2 and absorbance measured after one minute [20]. 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.

2.4.2. Protein determination

Protein concentration was quantified with Folin and Ciocalteu's phenol reagent following standard protocol while known concentrations of egg albumin (BDH) were use to extrapolate the standard curve [21]

2.4.3 Ammonium sulphate precipitation and dialysis

The extracts from flasks were centrifuged at 12500 r/min for 25 min, at 4 ^{o}C (Sigma, Germany Model 3K-30).The supernatant was subjected to ammonium sulphate precipitation in the range of 0 - 80 % (w/v) in an ice bath. The saturated solution was left overnight at 4 °C. Precipitated protein pellets were obtained by centrifugation as described above. The pellets collected were resuspended in 50 mL (50 mM, pH 4.5) sodium malonate buffer [22]. The concentrated sample with maximum laccase activity was dialyzed overnight against sodium malonate buffer (50 mM, pH 4.5) using dialysis tubing with Molecular Weigh Cut Off (MWCO) 12 - 14 kDa (Medical Intl. Ltd, 239 Liver Pool, London). The set up was left standing for the initial 2 hours after which the buffer was replaced with a fresh one and dialysis carried out for 24 hours [22]. Enzyme activity was determined before and after dialysis.

2.5 Characterization of Laccase

The enzyme was subjected to characterization through determination of the effect of the following on its activity:

2.5.1 Effect of pH on laccase activity

The effect of varying pH on laccase activity was carried out using different buffers and adjusted to different pH values ranging from 3.0, 4.0, 5.0, 6.0, 7.0, 8.5. Laccase was assayed at pH 3.0 in 20 mM Succinate buffer; pH 4.0 to 5.0 in 50 mM malonate buffer, pH 6.0 to 7.0 in 100 mM phosphate buffer, and pH 8.5 in 100 mM sodium phosphate buffer [23]. The enzyme assay was done using the protocol described earlier in 2.4.1.

2.5.2 Laccase stability under different pH values

Enzyme stability was determined by dispersing the enzyme (1:1) in 0.1 M buffer solutions pH 3.0 - 5.0 (sodium acetate), pH 5.0 - 7.0 (citrate-phosphate) and pH 7.0 - 8.5 (tris-HCl) and maintaining it at 25 ^{o}C for 24 hours. A 300 µL aliquot of the enzyme was used to determine the remaining activity at the optimum pH and temperature using standard assay protocol [18, 24].

2.5.3 Effect of temperature on laccase activity

The effect of varying temperature on laccase activity was carried out at different temperatures from 30 ^{o}C, 40 ^{o}C, 50 ^{o}C, 60 ^{o}C, 70 ^{o}C, 80 ^{o}C and 90 ^{o}C at optimum pH. The enzyme was incubated for 15 minutes and assayed by standard protocol [23].

2.5.4 Laccase thermo-stability

Enzyme stability under different temperature was evaluated by incubating the enzyme at various temperatures (20 ^{o}C - 90 ^{o}C) for 1 hour at optimum pH. A 300 µL aliquot of the enzyme was

withdrawn and placed on ice before assaying for remaining enzyme activity using standard assay protocol [18, 24].

2.5.5 Effect of metal ions on laccase activity.

To determine the effect of metal ions on enzyme activity, the reactions were performed by incubating the reaction mixture containing 300 µL of 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 ^{o}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 mM, 3 mM and 5 mM. After incubation the remaining enzyme activity was assayed. A heat-denatured enzyme was used as control [6, 25, 26].

2.5.6 Effect of ethylenediaminetetraacetic acid (EDTA) and L-cysteine on enzyme activity

The effect of EDTA and L-cysteine on laccase activity was monitored. To 1.4 mL of the reaction mixture, 800 µL of 0.1 M sodium acetate buffer containing ABTS (0.18 mM, pH 4.5), 300 µL of enzyme and 300 µL of inhibitor at various concentrations 1 mM - 5 mM were added. The reaction mixture was incubated at 30 ^{o}C for 30 minutes and the change in absorbance was measured using spectrophotometer at 436 nm. A control test was conducted in parallel in the absence of the inhibitor [6, 22].

2.5.7 Effect of substrate concentration: determination of K_m and V_{max}

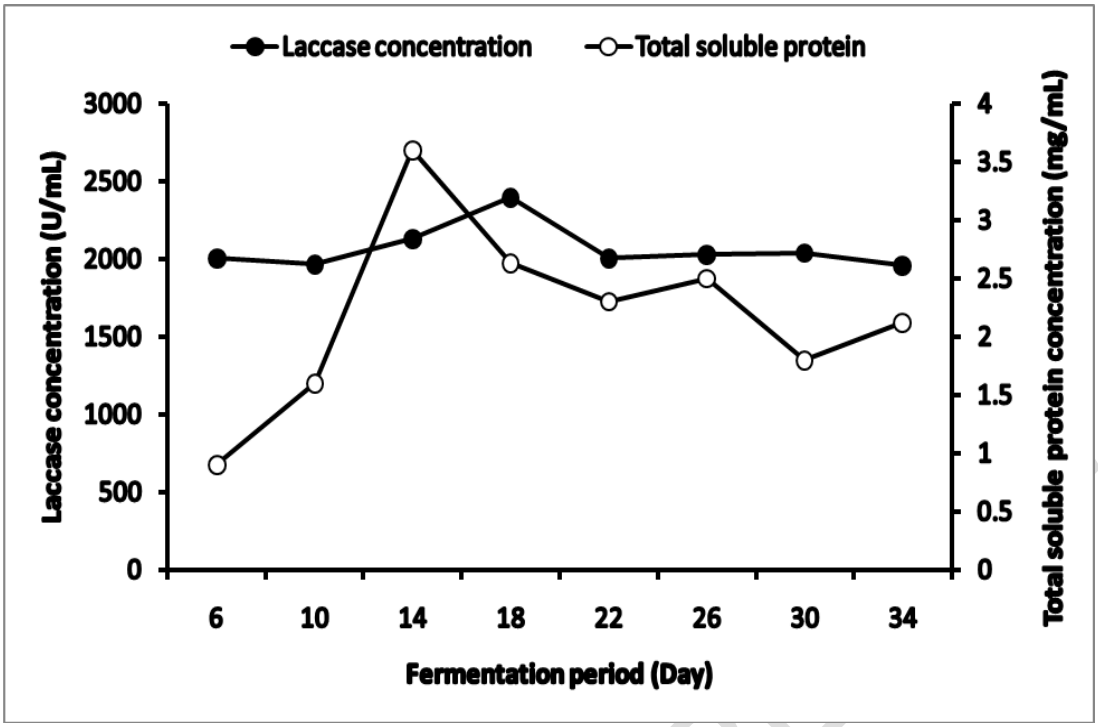
The Michalis-Menten kinetic parameters (K_m, V_{max}) were determine by measuring the laccase activity using varying concentrations of ABTS ranging from 0.1 mM to 0.5 mM. The parameter values were obtained by curve fitting of the reciprocal plot of reaction rate (V) versus substrate concentration (S) using Linweaver-Burk plot [27].

2.6 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 [28].

3. RESULTS AND DISCUSSION

Fig. 1. present the fermentation period and optimum pH for laccase production by *Trametes sp.* isolate B7 (GenBank accession number MK024175). The optimum fermentation period for Total Soluble Protein (TSP) and laccase production were day 14 and day 18 respectively in solid state fermentation of *Terminalia superba* sawdust (Fig. 1 a) while the optimum pH for TSP (3.6 mg/mL) and laccase (2356 U/mL) were produced by *Trametes sp*. isolate B7 at pH 5.0 (Fig. 1 b). Many investigators have reported different incubation periods for optimum production of crude laccases. Some authorities reported maximum laccase production on day 7 and day 10 of incubation using *Lentinus edodes* and *Ganoderma sp*. respectively while another work reported maximum laccase production on day 11 with rubber wood sawdust [29, 30, 31]. In another study, maximum production of laccase by *Ganoderma lucidum* was obtained on day 16 which was close to our finding [32]. Some authorities 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 [30, 33]. It has been established that the optimum pH for laccase production is dependent on the species and strain in addition to the lignocellulosic substrate [34]. The laccase was 2.3 and 9.0 times purified with specific activity of 1487 U/mL and 5380 U/mL for pellets and dialysate after ammonium sulphate precipitation and dialysis respectively as earlier described [16].



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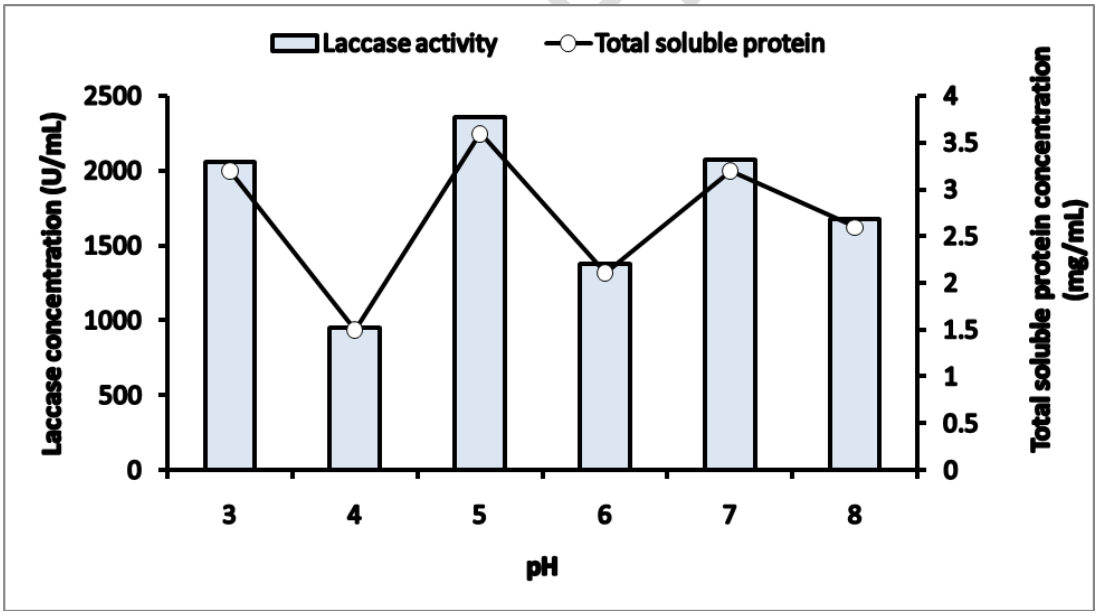
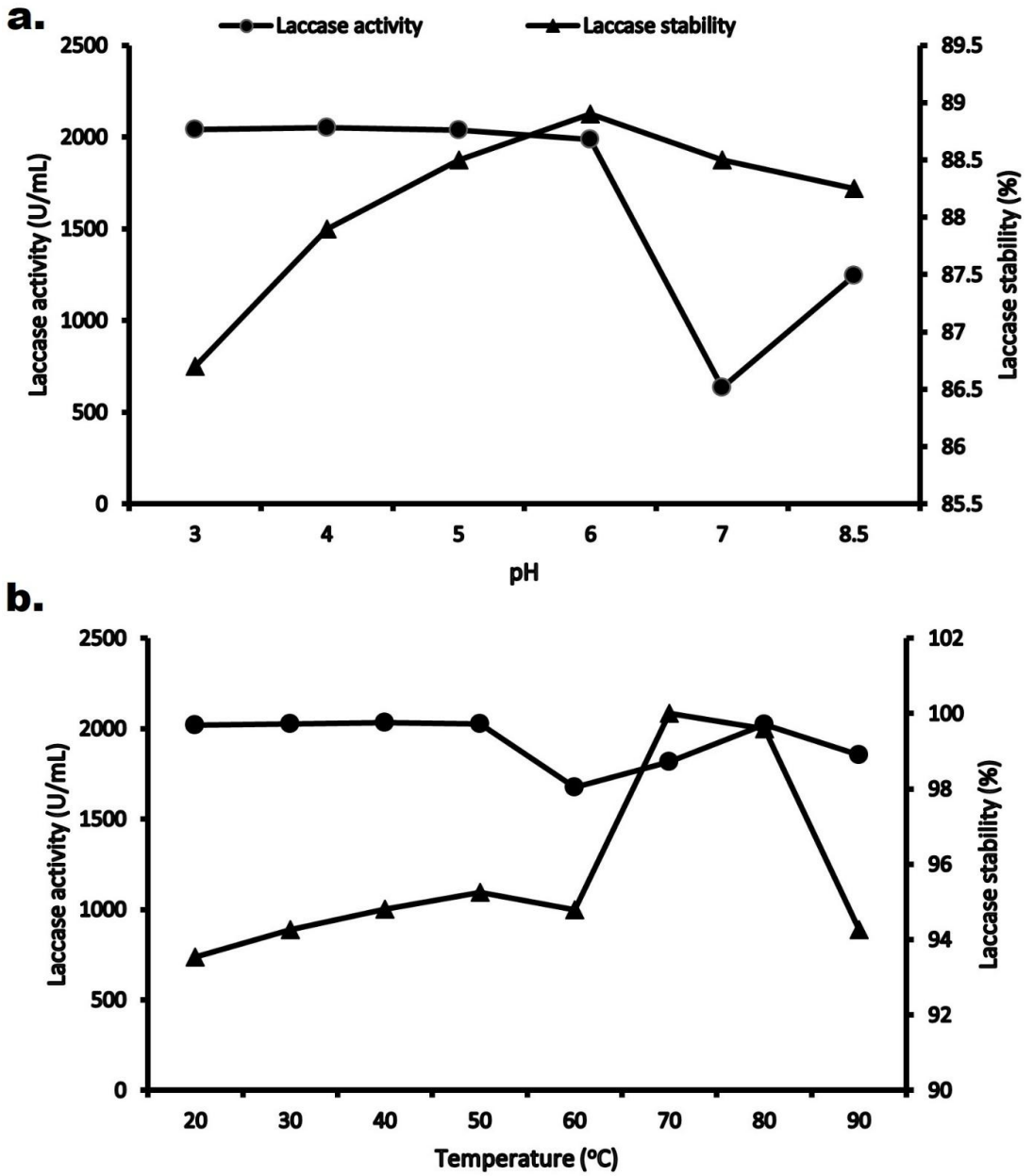


Figure 1: Laccase and total soluble protein production by *Trametes sp.* isolate B7 in the solid-state fermentation of *Terminalia superba* sawdust. a. Determination of optimum fermentation period of TSP (day 14) and laccase (day 18) at pH 5.0; b. Optimum pH for TSP and laccase production at pH 5.0.

The activity and stability of the partially purified laccase is presented in Fig. 2a. The partially purified laccase was active in the acidic pH 3.0 - 6.0. It has been reported that most fungal laccases are active in the acidic range of pH 3.0 - 4.0 [26]. For instance, *T. versicolor* laccase has optimum activity at pH

3.5 [35]. Another authority reported the characterization of extracellular laccases from *Fomes annosus*, *Pluerotus ostreatus*, *Trametes versicolor*, *Rhizoctonia praticola* and *Botrytis cinerea* and observed that the optimum activity varied between pH 3.0 - 5.0 which was close to this study [36]. The purified laccase had high stability in a pH range of 3.0 - 8.5 and with optimum stability of 89% at pH 6.0. This suggests that the enzyme may be useful in many biotechnological processes, especially those that require acidic conditions. This goes in line with a report that purified laccases of basidiomycete *Funalia trogii* (Berk.) exhibited broad pH activity and with optimum at pH 4.0 using 2, 6-dimethoxyphenol (DMP) as substrate [10]. These differences in peaks of optimum pH may be due to differences in fungal species as well as the substrates used in characterization of the enzymes.

The laccase was active and stable over a wide temperature range (Figure 2b). Laccase activity was high in the range of 20 ^{o}C - 50 ^{o}C and 80 ^{o}C. One authority reported the optimum temperature for maximum activity of laccase produced by *T. versicolor* as 40 °C [35] which falls within the range of 20 ^{o}C - 50 ^{o}C observed in this study. The enzyme was stable from 30 ^{o}C to 80 ^{o}C with an optimum stability of 100% at 70 ^{o}C. The enormous stability of this enzyme makes it more attractive for biotechnological and industrial applications. Other studies indicated 30 ^{o}C - 60 °C as optimal temperature range for the most fungal laccases and 55 ^{o}C - 65 ºC for laccases with thermophilic properties similar to thermostable laccases from basidiomycetes strains [18, 37].



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236 Figure 2 Effect of pH (a) and temperature (b) on partially purified *Trametes sp.* isolate B7 laccase
237 activity and stability.

238 EDTA was mildly inhibitory to laccase activity at 1 mM (0.55%), 3 mM (0.18%), 4 mM (2.92%) and 5
239 mM (6.70%) but activated laccase activity at 2 mM concentration with 117%. However, L-cysteine was
240 inhibitory to laccase activity at all the concentrations with 37.74%, 37.92%, 35.78%, 35.36% and
241 38.10% at 1 mM, 2 mM, 3 mM, 4 mM and 5 mM respectively (Figure 3a). EDTA is an inhibitor of
242 metallo-enzymes including laccase due to its property of forming inactive complexes with inorganic
243 prosthetic groups/co-factors of the enzyme [6]. It has been established that L-cysteine is a stronger
244 inhibitor of laccase activity than EDTA which agreed with our study [22]. The ability of the purified
245 enzyme to with stand the inhibitory effects of EDTA is important for industrial and biotechnological
246 processes which require enzymes that are resistance to metallic ions and other inhibitors especially
247 EDTA [5].

248 Five metal ions namely Mn^{2+}, Pb^{2+}, Hg^{2+}, Cu^{2+} and Mg^{2+} activated the partially purified laccase with
249 120.5%, 109%, 108%, 107.10% and 104% respectively (Figure 3b). The inhibitors were Al^{3+}, K^{2+}, Zn^{2+}
250 and $Fe^{2+}$with inhibitions of 35.20%, 34.30%, 34.20% and 27.33% respectively (Figure 3b). In the

presence of various salts, metallic ions affect enzymes substantially [26]. Report indicate that Mn^{2+},Mg^{2+} and K^{+} 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+} [26]. Another work reported higher inhibitory rates of 64% and 55% for Zn^{2+} and K^{+} respectively for purified laccase of *Lentinula edodes* [6]. This variation 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 [6].

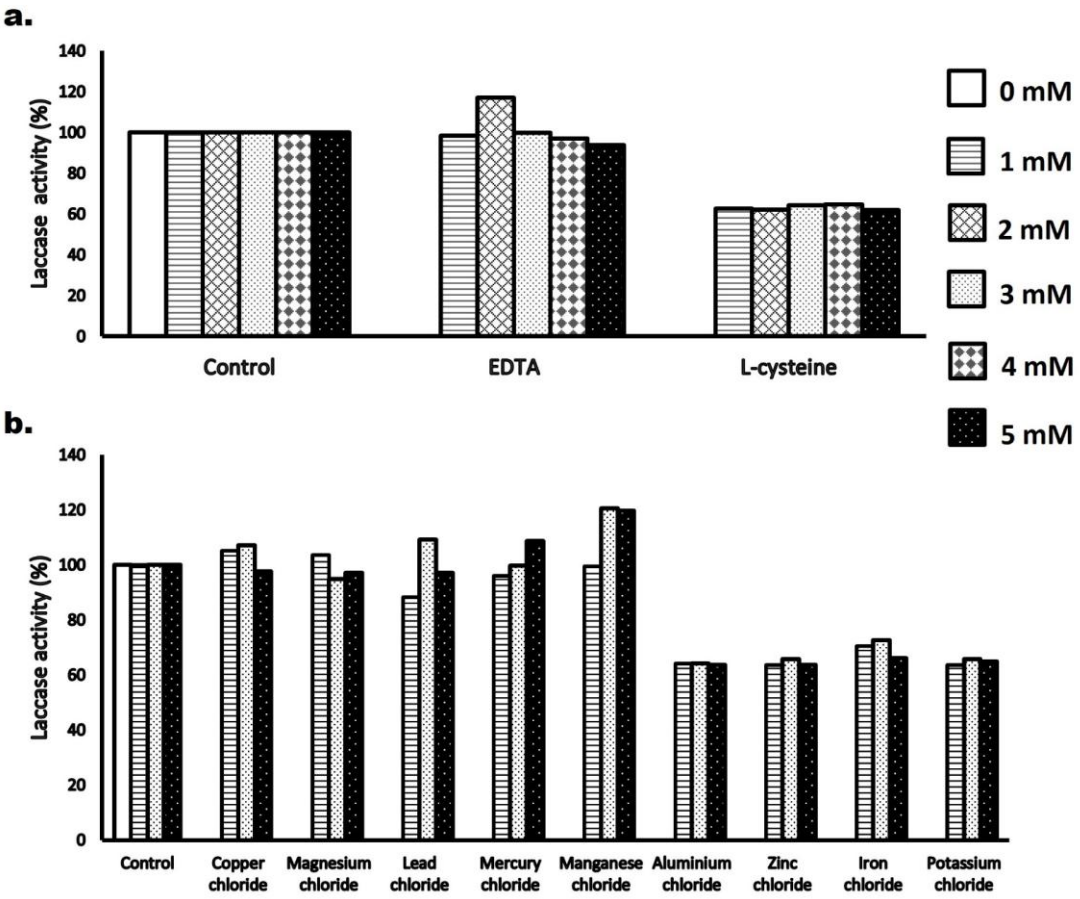

Figure 3 Effect of EDTA, L-cysteine and metal ions concentration on the activity of partially purified *Trametes sp.* isolate B7 laccase.

The purified laccase had Km 33 µM and Vmax 1.91 µMol./min/mL (Figure 4). The authors of one study reported Km 180 µM and Vmax 3.95 µmol/min/mg for purified laccase from *T. harzianum* [6] which implied that the partially purified laccase of *Trametes sp.* isolate B7 had a higher substrate affinity than that of *T. harzianum*. This is because the rate of reaction and concentration of substrate depends on its Km and the lower the Km the higher the substrate affinity [23]. A study to determine the molecular weight of the purified laccase showed a monomeric enzyme with molecular weight of ~36 kDa using one dimensional native gel electrophoresis as described [16]. The molecular weight was lower than other reported laccases in the range of 55 - 65 kDa, however; it was higher than that of *L. polychrous* with about 32 kDa [38]. Another study detected the presence of two iso-laccases with one of the bands at 38 kDa which is close in molecular weight to laccase of *Trametes sp.* isolate B7 [39].

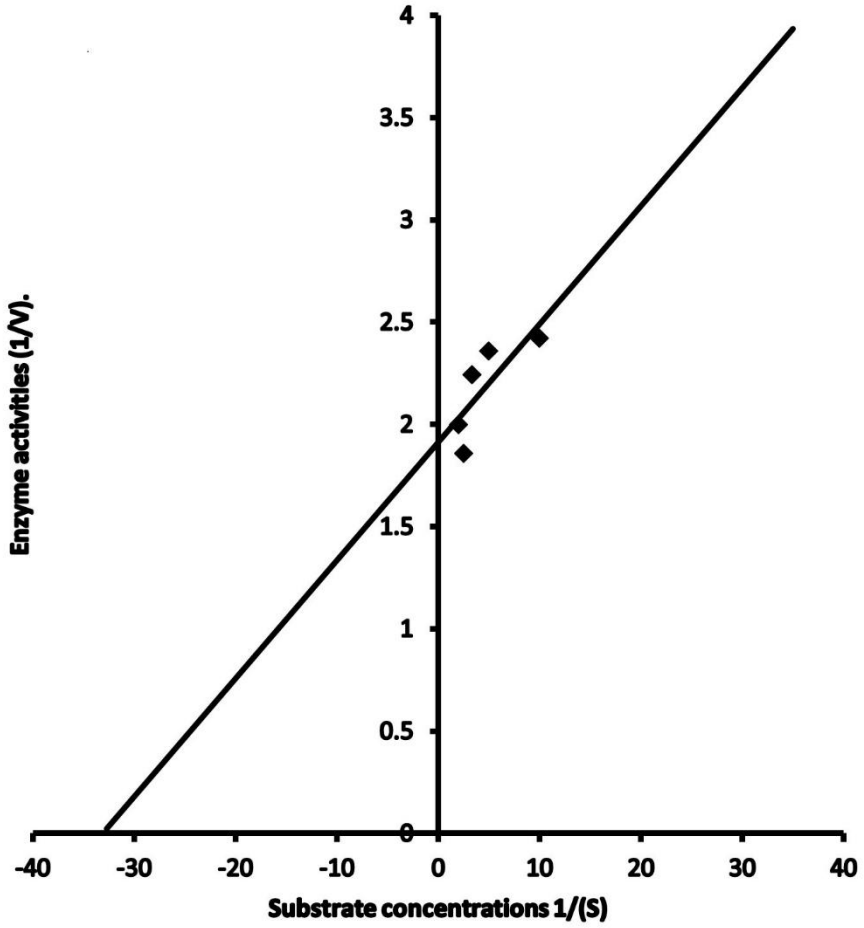

Figure 4 The kinetic constants (Km) and maximum rate of reaction (Vmax) of partially purified *Trametes sp.* isolate B7 laccase.

4. CONCLUSION

The study established sawdust as an alternative low cost substrate for production of cheap laccases utilizing *Trametes sp.* isolate B7. Production of the laccase was optimum at pH 5.0 on day 18. Purification and characterization of laccase showed high activity in acidic pH and a broad stability range. In addition it possessed high activities at high temperature and thermostable at 70 ^{o}C for 1 hr along with resistance to most metallic ions tested and EDTA. These attributes made the enzyme a potential tool for many biotechnological and industrial applications including those of pulp and mill paper, polycyclic aromatic hydrocarbons, pesticides and dyes waste waters among other xenobiotics.

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