Original Research Article

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

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ABSTRACT

Laccases catalyze a broad range of substrates due to their low substrate specificity and strong oxidative potentials. It can produce from different ~~S~~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 activity (2356 U/mL) were obtained~~produced~~ on day 14 and ~~day 1~~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 temperature at 20~~ °C - 50 °C and~~ 80 °C while, stability was highest at pH 6.0 (89% for 24 hr) and 70 °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 K_M 33 µM and V_{max} 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 wastewaters 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]. It has been estimated that about 140 billion tons of lignocellulosic feedstock are generated from the agricultural sector annually world-wide [2] with a substantial part considered as 'waste'. Large amounts of lignocellulosics wastes including leaves, roots, stalks, bark, bagasse, straw residues, seeds, and wood residues are produced through ~~many~~ agricultural, agro-based and forestry practices which constitute not only problem of disposal but also loss of valuable materials. In addition, primary and secondary processing generates unpreventable food supply chain waste especially in some developing countries of Africa with up to 75% losses during post harvest processing [2].

Lignocellulosic biomass, a non-food source, is a sustainable and natural resource-based biopolymer with high advantages over starch and sugar crops because it does not interfere with food and feed chain supplies [3]. Approximately 90% of lignocellulosic biomass consists of cellulose (30% - 60%), hemicelluloses (20% - 40%) and lignin (10% - 25%), whereas the rest comprises of ash and extractives [4]. Cellulose is the most abundant biopolymer on earth and has received much attention as a renewable resource for bioconversion to value added products of commercial importance [5]. However, access to the sugar component is a major challenge in biorefining of lignocellulosics to biofuels, biocatalysts and other chemicals of industrial importance [6]. This is because lignin confers a protective cover against chemical and enzymatic hydrolysis of cellulosic and hemicellulosic components of plant biomass [6].

The need for suitable pre-treatment techniques to eliminate or reduce lignin and expose cellulose and hemicelluloses for fermentation is therefore imperative [7]. Pre-treatment of lignocellulosic biomass may involve physical, chemical, biological or a combination of these methods. Chemical pre-treatment is the most common method and involves the use of acids, bases, ionic liquids and organic solvents in pre-treatment of plant biomass [8]. Biological pre-treatment utilize whole cell organisms or their enzymes to degrade lignin content of lignocellulose with significant reduction in loss of carbohydrates and is less expensive [9]. Therefore, it is very important to select microorganisms with high delignification potential and less ability to break down cellulose and hemicelluloses during pre-treatment. It has been reported that microbial delignification could increase carbohydrate content and saccharification efficiency of lignocelluosic biomass by 97.8% [6]. White rot fungi are very efficient in biological pre-treatment due to production of ligninolytic enzymes which break down lignin [10]. However, inadequate production of ligninolytic enzymes, low enzyme activity and stability of synthesized enzymes are critical factors that prevent their utilization in biotechnology [11].

The desire for industrially relevant enzymes has increased due to array of applications in various areas of industry and biotechnology. Lignases (laccase, lignin peroxidase and manganese peroxidase), cellulases, xylanases, pectinases and proteases are produced by different microorganisms using lignocellulosic biomass in Solid State Fermentation (SSF) [4]. Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are multicopper blue oxidases widely distributed in higher plants, some insects, a few bacteria, lichen and fungi [12, 13]. However, the best known laccases are of fungal origin occurring in various fungi over a wide range of taxa. Well known laccase producers include *Trametes versicolor, Chaetomium thermophilum*, *Agaricus bisporus*, *Botrytis cinerea*, *Coprinus cinereus*, *Phlebia radiata* and *Pleurotus eryngii* [14, 15]. Production of fungal laccases using plant biomass in SSF is an attractive alternative process due to its lower capital investment and lower operating cost [16].

Laccases are either monomeric or multimeric glycoproteins and their heterogeneity is dependent on variations in carbohydrate content or differences in copper content [17]. They also show considerable diversity in substrate specificity, molecular weight, temperature and pH optimum as well as other properties depending on the organism [18, 19]. Due to their low substrate specificity and strong oxidative abilities, laccases have a variety of industrial applications in dough or baked products to increase strength of gluten structures, pharmaceutical industries as anesthetics, anti-inflammatory drugs, antibiotics, and sedatives [20, 21], animal feed, clinical diagnosis enzyme immunoassays, detoxification of environmental pollutants, biopulping, textile dye bleaching, removal of herbicides from cereal crops, and enzymatic conversion of chemical intermediates [22, 23]. In addition, it is used in fast moving consumer goods (FMCG) such as tooth-paste, mouthwash, detergent, soap, and diapers in cosmetics, as deodorants; in beverage and food industry for wine and juice stabilization [21]. Nevertheless, high costs of production, low enzyme activities and stabilities have limited large-scale applications of laccases in areas of industry and biotechnology [23, 24].

The objective of this study was to produce novel laccase from *Trametes sp.* isolate B7 with high activity and stability over a wide pH range and high temperatures, resistant to inhibitory effects of metallic ions and EDTA that exist in large quantities during industrial processes, and capable of industrial or biotechnological applications using cheap substrate such as *Terminalia superba* sawdust in SSF.

2. MATERIALS AND METHODS

2.1 Substrate Collection and Preparation

Wood samples of *Terminalia superba* Engl. & 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 [25]. 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 [26]. The fungal isolate was identified using molecular and phylogenetic characterization as earlier described [27]. 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 [28]. Ten milliliters 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 [29].

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 [30].

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 [31]. 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 [32].

2.4.3 Ammonium sulphate precipitation and dialysis

The extracts from flasks were centrifuged at 17150 RCF/G for 20 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 reconstituted in 50 mL (50 mM, pH 4.5) sodium malonate buffer [33]. The concentrated sample with maximum laccase activity was dialyzed overnight against sodium malonate buffer (50 mM, pH 4.5) using dialysis tubing with Molecular Weight 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 [33]. Enzyme activity was determined before and after dialysis.

2.5 Characterization of Laccase

The enzyme was characterized by determining the effects of various parameters on its activity and stability. The effect of pH on laccase activity was determined by adjusting different buffers to pH values ranging from 3.0 - 8.5. 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 [34] following the standard protocol described in 2.4.1. Laccase stability was determined by incubating 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) at 25 ^{o}C for 24 hours. A 300 µL aliquot was used to determine the remaining activity at optimum pH and temperature [29, 35]. The effect of temperature on laccase activity was carried out at 30 ^{o}C - 90 ^{o}C for 15 minutes at optimum pH following the standard protocol [34]. Laccase stability was evaluated at 20 ^{o}C - 90 ^{o}C for 1 hour using optimum pH. A 300 µL aliquot enzyme was withdrawn and placed on ice before assaying for remaining activity [29, 35].

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 ^{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 [17, 36, 37]. 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, pH 4.5), 300 µL of enzyme and 300 µL of inhibitor at various concentrations 1 mM - 5 mM. Incubation was at 30 ^{o}C for 30 minutes and the absorbance measured at 436 nm using spectrophotometer. A control test was conducted in the absence of the inhibitor [17, 33].

The Michalis-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 [38].

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 [39].

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 authors 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 [22, 40, 41]. In another study, maximum production of laccase by *Ganoderma lucidum* was obtained on day 16 which was close to our finding [42]. Some 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 [41, 43]. It has been established that the optimum pH for laccase production is dependent on the species and strain in addition to the lignocellulosic substrate [44]. 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 [27].

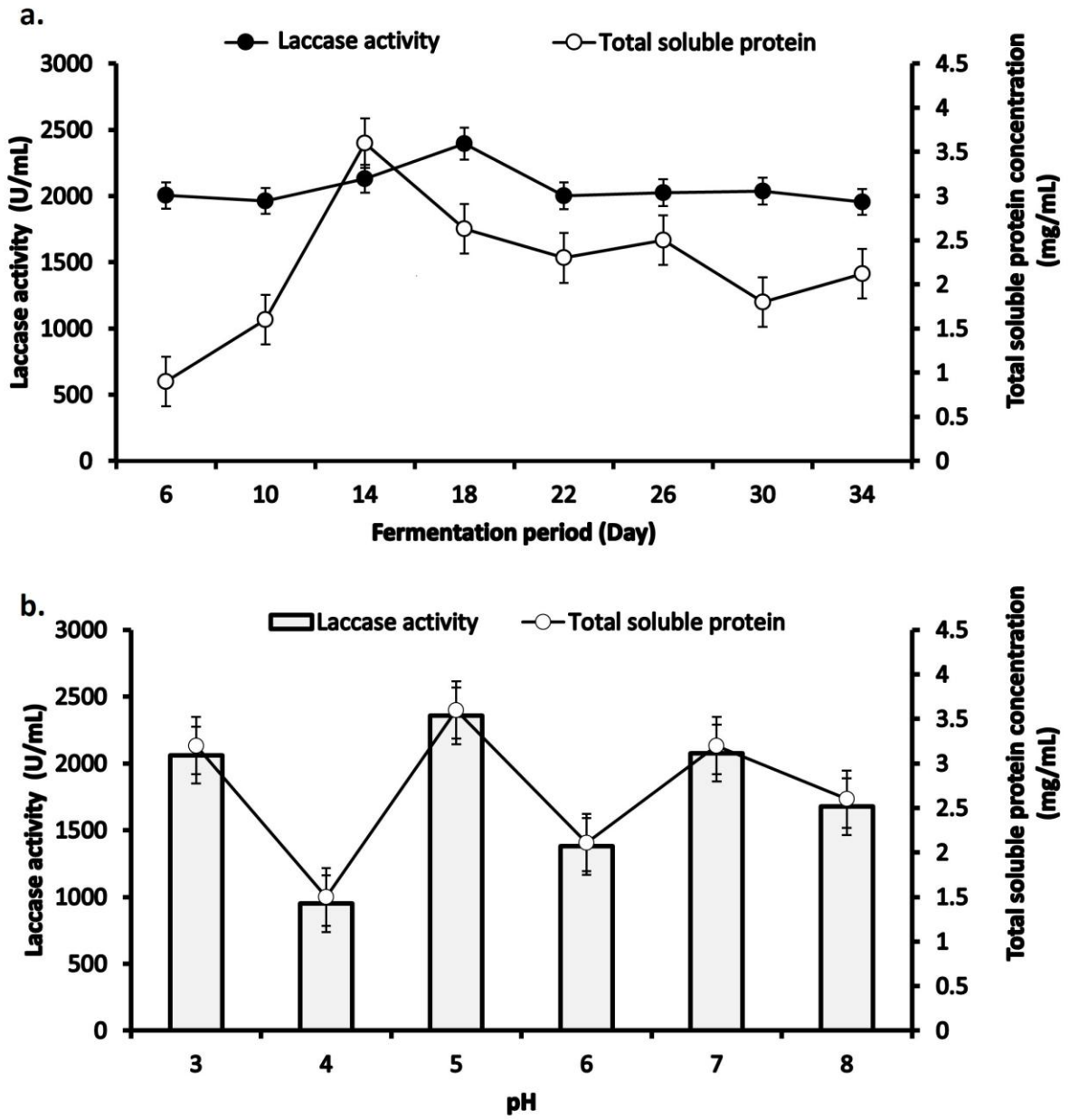

Figure 1 Laccase and total soluble protein production by *Trametes sp.* isolate B7 in the solid-state fermentation of *Terminalia superba* sawdust. a. Determination of optimum fermentation period of TSP (day 14) and laccase (day 18) at pH 5.0; b. Optimum pH for TSP and laccase production at pH 5.0. Bar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

The activity and stability of the partially purified laccase are 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 [37]. For instance, *T. versicolor* laccase has optimum activity at pH 3.5 [45]. Another author 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 [46]. 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.) Bondertsev & Singer exhibited broad pH activity and with optimum at pH 4.0 using 2, 6-dimethoxyphenol (DMP) as substrate [47]. 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 author reported the optimum temperature for maximum activity of laccase produced by *T. versicolor* as 40 °C [45] 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 most fungal laccases and 55 ^{o}C - 65 ºC for laccases with thermophilic properties similar to thermostable laccases from basidiomycetes strains [29, 48].

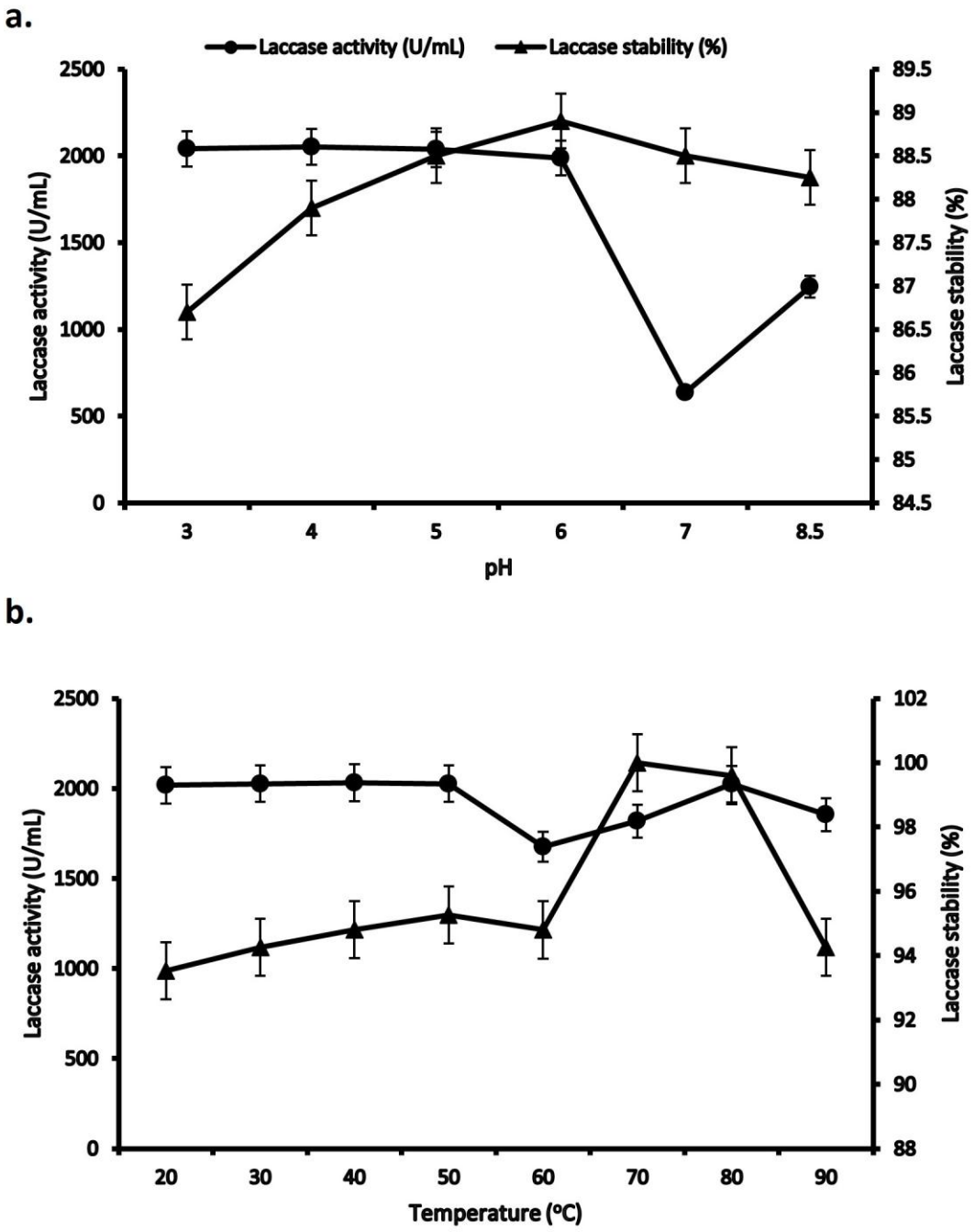

Figure 2 Effect of pH (a) and temperature (b) on partially purified *Trametes sp.* isolate B7 laccase activity and stability. Bar represent standard error of duplicate determination.

EDTA was mildly inhibitory to laccase activity at 1 mM (0.55%), 3 mM (0.18%), 4 mM (2.92%) and 5 mM (6.70%) but activated laccase activity at 2 mM concentration with 117%. However, L-cysteine was inhibitory to laccase activity at all the concentrations with 37.74%, 37.92%, 35.78%, 35.36% and 38.10% at 1 mM, 2 mM, 3 mM, 4 mM and 5 mM respectively (Figure 3a). 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 [17]. It has been established that L-cysteine is a stronger inhibitor of laccase activity than EDTA which agreed with our study [33]. The ability of the purified enzyme to with stand the inhibitory effects of EDTA is important for industrial and biotechnological processes which require enzymes that are resistance to metallic ions and other inhibitors especially EDTA [14].

Five metal ions namely Mn^{2+}, Pb^{2+}, Hg^{2+}, Cu^{2+} and Mg^{2+} activated the partially purified laccase with 120.5%, 109%, 108%, 107.10% and 104% respectively (Figure 3b). The inhibitors were Al^{3+}, K^{2+}, Zn^{2+} 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 [37]. 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+} [37]. Another work reported higher inhibitory rates of 64% and 55% for Zn^{2+} and K^{+} respectively for purified laccase of *Lentinula edodes* [17]. 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 [17].

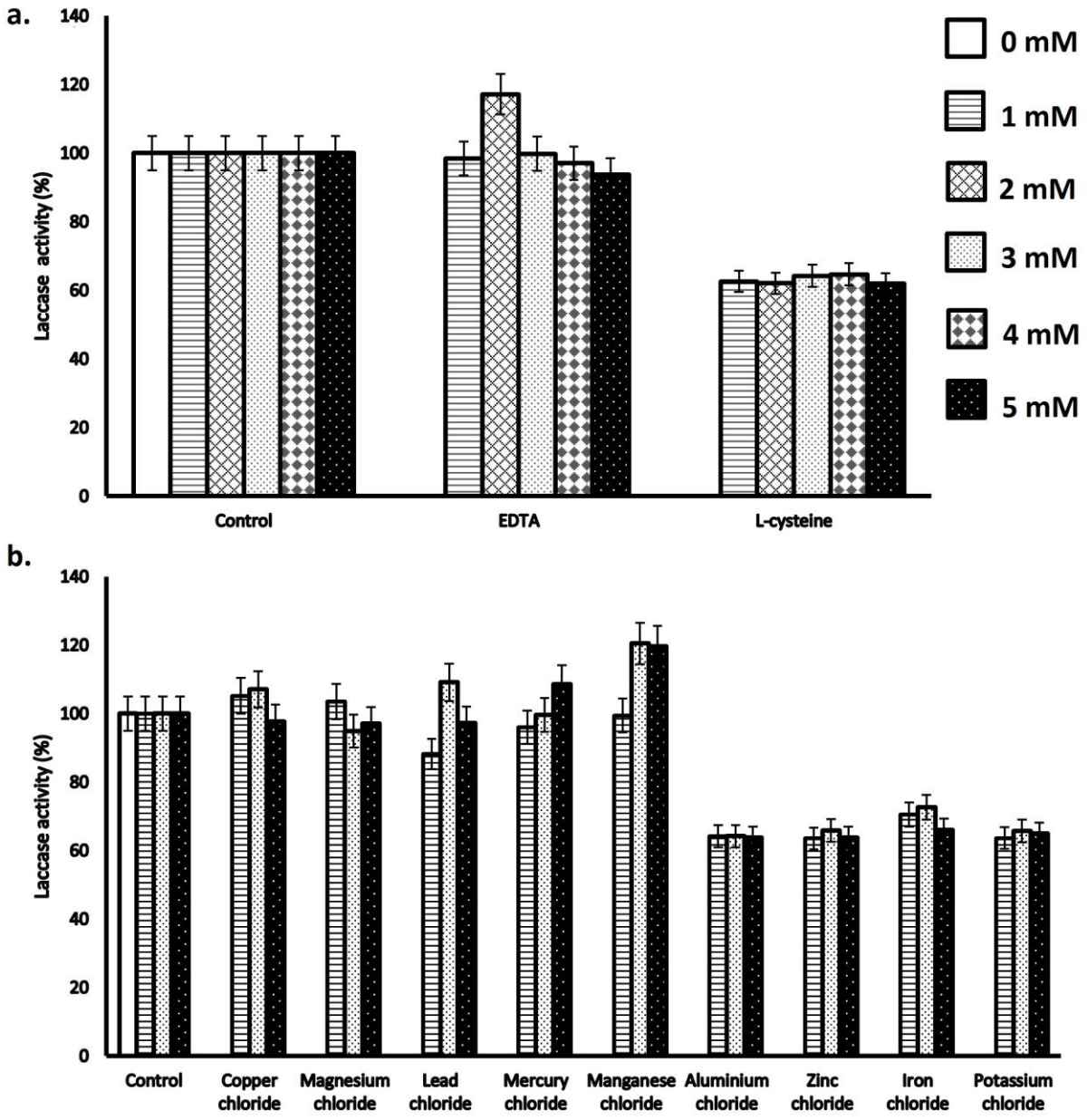

Figure 3 Effect of (a) EDTA, L-cysteine and (b) metal ions concentration on the activity of partially purified *Trametes sp.* isolate B7 laccase. Bar represent standard error of duplicate determination.

The purified laccase had K_M 33 µM and V_{max} 1.91 µMol./min/mL (Figure 4). The authors of one study reported K_M 180 µM and V_{max} 3.95 µmol/min/mg for purified laccase from *T. harzianum* [17] 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 K_M and the lower the K_M the higher the substrate affinity [34]. 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 [27]. The molecular weight was lower than other laccases which were reported in the range of 55 - 65 kDa, however; it was higher than that of *L. polychrous* with about 32 kDa [49]. Another study detected the presence of two iso-laccases with one of the bands at 38 kDa which is close in molecular weight to that of *Trametes sp.* isolate B7 laccase [50].

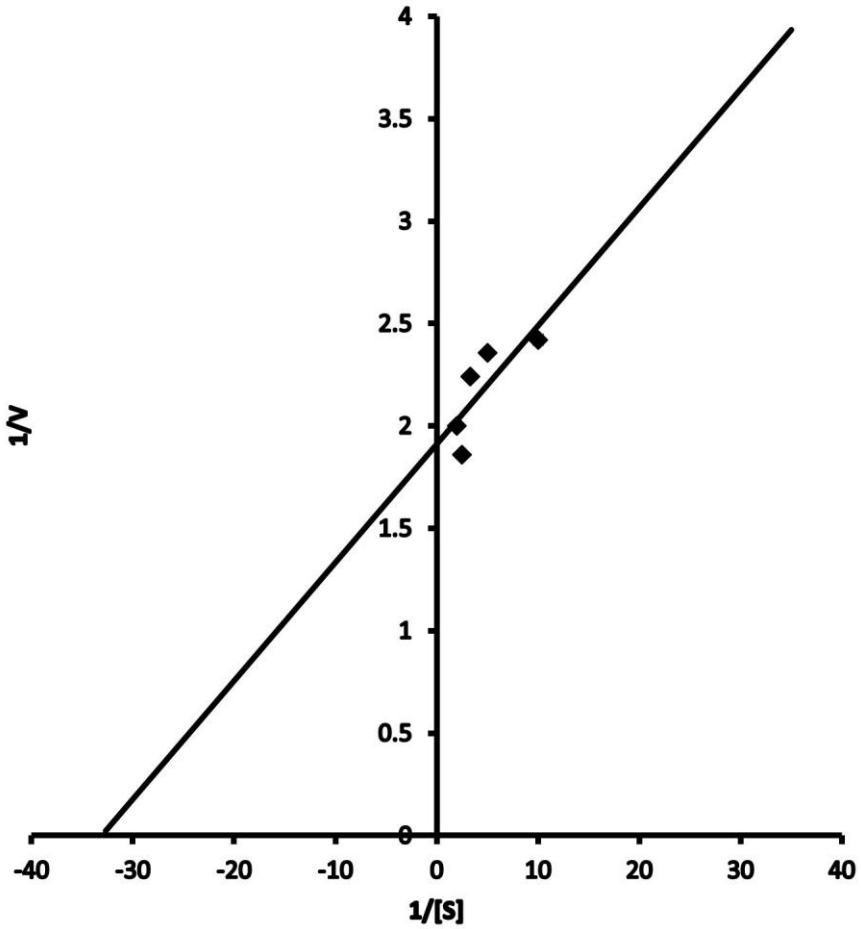

Figure 4 Lineweaver-Burk reciprocal plot: Determination of K_M and V_{max} of purified laccase from *Trametes sp.* isolate B7. 1/V represent (Velocity of reaction) and 1/[S] (Substrate concentration).

4. CONCLUSION

The study established *Terminalia superba* 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. Characterization of laccase showed high enzyme activity in acidic pH and a broad enzyme stability in acidic to mild alkaline range. In addition it was active at high temperature, thermostable at 70 ^{o}C for 1 hr and resistant to most metallic ions 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 wastewaters among other xenobiotics.

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