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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](#) 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](#) on day 14 and 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 - 6.0](#)) and [temperature](#) at 20-80 °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 μ M and V_{max} 1.91 μ Mol./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 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].

39 The need for suitable pre-treatment techniques to eliminate or reduce lignin and expose cellulose and
40 hemicelluloses for fermentation is therefore imperative [7]. Pre-treatment of lignocellulosic biomass
41 may involve physical, chemical, biological or a combination of these methods. Chemical pre-treatment
42 is the most common method and involves the use of acids, bases, ionic liquids and organic solvents
43 in pre-treatment of plant biomass [8]. Biological pre-treatment utilize whole cell organisms or their
44 enzymes to degrade lignin content of lignocellulose with significant reduction in loss of carbohydrates
45 and is less expensive [9]. Therefore, it is very important to select microorganisms with high
46 delignification potential and less ability to break down cellulose and hemicelluloses during pre-
47 treatment. It has been reported that microbial delignification could increase carbohydrate content and
48 saccharification efficiency of lignocellulosic biomass by 97.8% [6]. White rot fungi are very efficient in
49 biological pre-treatment due to production of ligninolytic enzymes which break down lignin [10].
50 However, inadequate production of ligninolytic enzymes, low enzyme activity and stability of
51 synthesized enzymes are critical factors that prevent their utilization in biotechnology [11].
52

53 The desire for industrially relevant enzymes has increased due to array of applications in various
54 areas of industry and biotechnology. Lignases (laccase, lignin peroxidase and manganese
55 peroxidase), cellulases, xylanases, pectinases and proteases are produced by different
56 microorganisms using lignocellulosic biomass in Solid State Fermentation (SSF) [4]. Laccases
57 (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are multicopper blue oxidases widely distributed
58 in higher plants, some insects, a few bacteria, lichen and fungi [12, 13]. However, the best known
59 laccases are of fungal origin occurring in various fungi over a wide range of taxa. Well known laccase
60 producers include *Trametes versicolor*, *Chaetomium thermophilum*, *Agaricus bisporus*, *Botrytis*
61 *cinerea*, *Coprinus cinereus*, *Phlebia radiata* and *Pleurotus eryngii* [14, 15]. Production of fungal
62 laccases using plant biomass in SSF is an attractive alternative process due to its lower capital
63 investment and lower operating cost [16].
64

65 Laccases are either monomeric or multimeric glycoproteins and their heterogeneity is dependent on
66 variations in carbohydrate content or differences in copper content [17]. They also show considerable
67 diversity in substrate specificity, molecular weight, temperature and pH optimum as well as other
68 properties depending on the organism [18, 19]. Due to their low substrate specificity and strong
69 oxidative abilities, laccases have a variety of industrial applications in dough or baked products to
70 increase strength of gluten structures, pharmaceutical industries as anesthetics, anti-inflammatory
71 drugs, antibiotics, and sedatives [20, 21], animal feed, clinical diagnosis enzyme immunoassays,
72 detoxification of environmental pollutants, biopulping, textile dye bleaching, removal of herbicides
73 from cereal crops, and enzymatic conversion of chemical intermediates [22, 23]. In addition, it is used
74 in fast moving consumer goods (FMCG) such as tooth-paste, mouthwash, detergent, soap, and
75 diapers in cosmetics, as deodorants; in beverage and food industry for wine and juice stabilization
76 [21]. Nevertheless, high costs of production, low enzyme activities and stabilities have limited large-
77 scale applications of laccases in areas of industry and biotechnology [23, 24].
78

79 The objective of this study was to produce novel laccase from *Trametes sp.* isolate B7 with high
80 activity and stability over a wide pH range and high temperatures, resistant to inhibitory effects of
81 metallic ions and EDTA that exist in large quantities during industrial processes, and capable of
82 industrial or biotechnological applications using cheap substrate such as *Terminalia superba* sawdust
83 in SSF.
84

85 2. MATERIALS AND METHODS

86

87 2.1 Substrate Collection and Preparation

88

89 Wood samples of *Terminalia superba* Engl. & Diels were collected from Gboko plank market, Benue
90 State, North-Central Nigeria. The samples were passed through an electric sliding-table saw machine
91 to obtain wood blocks which were oven dried to constant weight at 80 °C. The blocks were directly fed
92 into a motorized rotary machine and crushed into sawdust particles. The sawdust was then passed
93 through a 2 mm wire mesh of metallic sieve to obtain particles of even sizes so that fungal growth
94 would not differ due to differences in oxygen diffusion, nutrient absorption and assimilation by mycelia
95 [25]. The substrate was then dispensed into and sealed in plastic bags and stored in the laboratory.

96

97 2.2 Isolation and Identification of Fungal strain

98

99 The fungus used in this study was isolated from a decaying wood in Benue Polytechnic Campus,
100 Ugbokolo, Benue State, Nigeria. Pieces of sample were placed on fully sterile Potato Dextrose Agar
101 (PDA) plates and incubated at 27 °C ± 2 °C for 7 days. Pure cultures were obtained by sub-culturing
102 onto fresh sterile PDA plates and placed on PDA slants which were refrigerated at 4 °C. Five-day old
103 fungal cultures on PDA plates were observed for both cultural and morphological characteristics [26].
104 The fungal isolate was identified using molecular and phylogenetic characterization as earlier
105 described [27]. The sequence was deposited in GenBank under the accession number MK024175.

106

107 2.3 Media and Culture Conditions

108

109 The Lignin Modifying Medium (LMM) used to moisten the sawdust sample was adjusted to pH 3.0 -
110 8.0 and had the following composition (g L⁻¹) glucose 10 g, Ammonium tartrate 2 g, KH₂PO₄ 1 g,
111 MgSO₄·7H₂O 0.5 g, KCl 0.5 g, Yeast extract 1 g, Soy tone 5 g, CuSO₄·5H₂O (150 µm), EDTA 0.5 g,
112 FeSO₄ 0.2 g, ZnSO₄ 0.0 1g, MnCl₂·4H₂O 0.00 3g, H₃BO₄ 0.03 g, CoCl₂·6H₂O 0.02 g, CuCl₂·2H₂O
113 0.001 g, Na₂MoO₄·2H₂O 0.003 g [28]. Ten milliliters of the medium was added to 100 g of the sawdust
114 with approximately 70% moisture content in 250 mL Erlenmeyer flask and sterilized by autoclaving at
115 121 °C for 20 minutes. One percent (w/v) aqueous glucose solution was separately autoclaved at 110
116 °C (10 psi) for 10 minutes and 2 mL aseptically added to the fermenting flask. Flasks were allowed to
117 cool then aseptically inoculated with two 5 mm agar plugs of actively growing mycelia from a 5-day old
118 fungi culture on PDA. Flasks were prepared in duplicate and incubated at 27 °C ± 2 °C for
119 6,10,14,18,22,26,30 and 34 days [29].

120

121 2.4 Extraction of Extracellular Enzymes

122

123 Extracellular enzymes were extracted by addition of 100 mL of 0.1M citrate-phosphate buffer (pH 5.0)
124 into the fermenting flask. The mixture was stirred with a glass rod for 30 minutes and filtered with
125 cheese-cloth to remove sawdust and fungal mycelia. The crude filtrate was then filtered with 90 mm
126 Whatman No. 1 Filter paper to obtain a clear filtrate which was refrigerated at 4 °C [30].

127

128 2.4.1 Assay of laccase activity

129

130 Laccase activity was determined at 420 nm with Spectrophotometer using 2, 2'-azino-bis (3-
131 ethylbenz-thiazoline-6-sulfonic acid (ABTS). The reaction mixture consisted of 600 µL sodium acetate
132 buffer (0.1 M, pH 5.0 at 27 °C), 300 µL ABTS (5 mM), 300 µL culture supernatant and 1400 µL
133 distilled water. The reaction was incubated for 2 minutes at 30 °C and initiated by adding 300 µL H₂O₂
134 and absorbance measured after one minute [31]. One Unit of laccase activity was defined as activity
135 of an enzyme that catalyzes the conversion of 1 µmol of ABTS (ε=36,000 M⁻¹ cm⁻¹) per minute.

136

137 2.4.2 Protein determination

138

139 Protein concentration was quantified with Folin and Ciocalteu's phenol reagent following standard
140 protocol while known concentrations of egg albumin (BDH) were use to extrapolate the standard
141 curve [32].

142

143 2.4.3 Ammonium sulphate precipitation and dialysis

144

145 The extracts from flasks were centrifuged at 17150 RCF/G for 20 min, at 4 °C (Sigma, Germany
146 Model 3K-30). The supernatant was subjected to ammonium sulphate precipitation in the range of 0 -
147 80% (w/v) in an ice bath. The saturated solution was left overnight at 4 °C. Precipitated protein pellets
148 were obtained by centrifugation as described above. The pellets collected were reconstituted in 50 mL
149 (50 mM, pH 4.5) sodium malonate buffer [33]. The concentrated sample with maximum laccase
150 activity was dialyzed overnight against sodium malonate buffer (50 mM, pH 4.5) using dialysis tubing
151 with Molecular Weight Cut Off (MWCO) 12 - 14 kDa (Medical Intl. Ltd, 239 Liver Pool, London). The
152 set up was left standing for the initial 2 hours after which the buffer was replaced with a fresh one and
153 dialysis carried out for 24 hours [33]. Enzyme activity was determined before and after dialysis.

154

155 2.5 Characterization of Laccase

156

157 The enzyme was characterized by determining the effects of various parameters on its activity and
158 stability. The effect of pH on laccase activity was determined by adjusting different buffers to pH
159 values ranging from 3.0 - 8.5. Laccase activity at pH 3.0 was assayed in 20 mM Succinate buffer, pH
160 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
161 sodium phosphate buffer [34] following the standard protocol described in 2.4.1. Laccase stability was
162 determined by incubating the enzyme (1:1) in 0.1 M buffer solutions pH 3.0 - 5.0 (sodium acetate), pH
163 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
164 used to determine the remaining activity at optimum pH and temperature [29, 35]. The effect of
165 temperature on laccase activity was carried out at 30 °C - 90 °C for 15 minutes at optimum pH
166 following the standard protocol [34]. Laccase stability was evaluated at 20 °C - 90 °C for 1 hour using
167 optimum pH. A 300 µL aliquot enzyme was withdrawn and placed on ice before assaying for
168 remaining activity [29, 35].

169

170 The effect of metal ions on laccase activity was determined by incubating the reaction mixture of 300
171 µL enzyme, 800 µL of 0.1 M sodium acetate buffer containing ABTS (0.18 mM, pH 4.5) and 300 µL
172 metal ion solution at 30 °C for 30 minutes. The metal ions Cu²⁺, Mg²⁺, Pb²⁺, Hg²⁺, Mn²⁺, Al³⁺, Zn²⁺,
173 Fe²⁺ and K⁺ in their chloride forms were used at the concentration of 1 mM, 3 mM and 5 mM. After
174 incubation, the remaining enzyme activity was assayed. A heat-denatured enzyme was used as
175 control [17, 36, 37]. The effect of EDTA and L-cysteine on laccase activity was determined by
176 incubating 1.4 mL reaction mixture comprising 800 µL of 0.1 M sodium acetate buffer containing
177 ABTS (0.18 mM, pH 4.5), 300 µL of enzyme and 300 µL of inhibitor at various concentrations 1 mM -
178 5 mM. Incubation was at 30 °C for 30 minutes and the absorbance measured at 436 nm using
179 spectrophotometer. A control test was conducted in the absence of the inhibitor [17, 33].

180

181 The Michalis-Menten kinetic parameters (K_M , V_{max}) were determined by measuring laccase activity at
182 varying concentrations of ABTS from 0.1 mM - 0.5 mM. The parameters were obtained by curve fitting
183 the reciprocal plot of reaction rate (V) versus substrate concentration (S) using Lineweaver-Burk plot
184 [38].

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186 2.6 Statistical Analysis

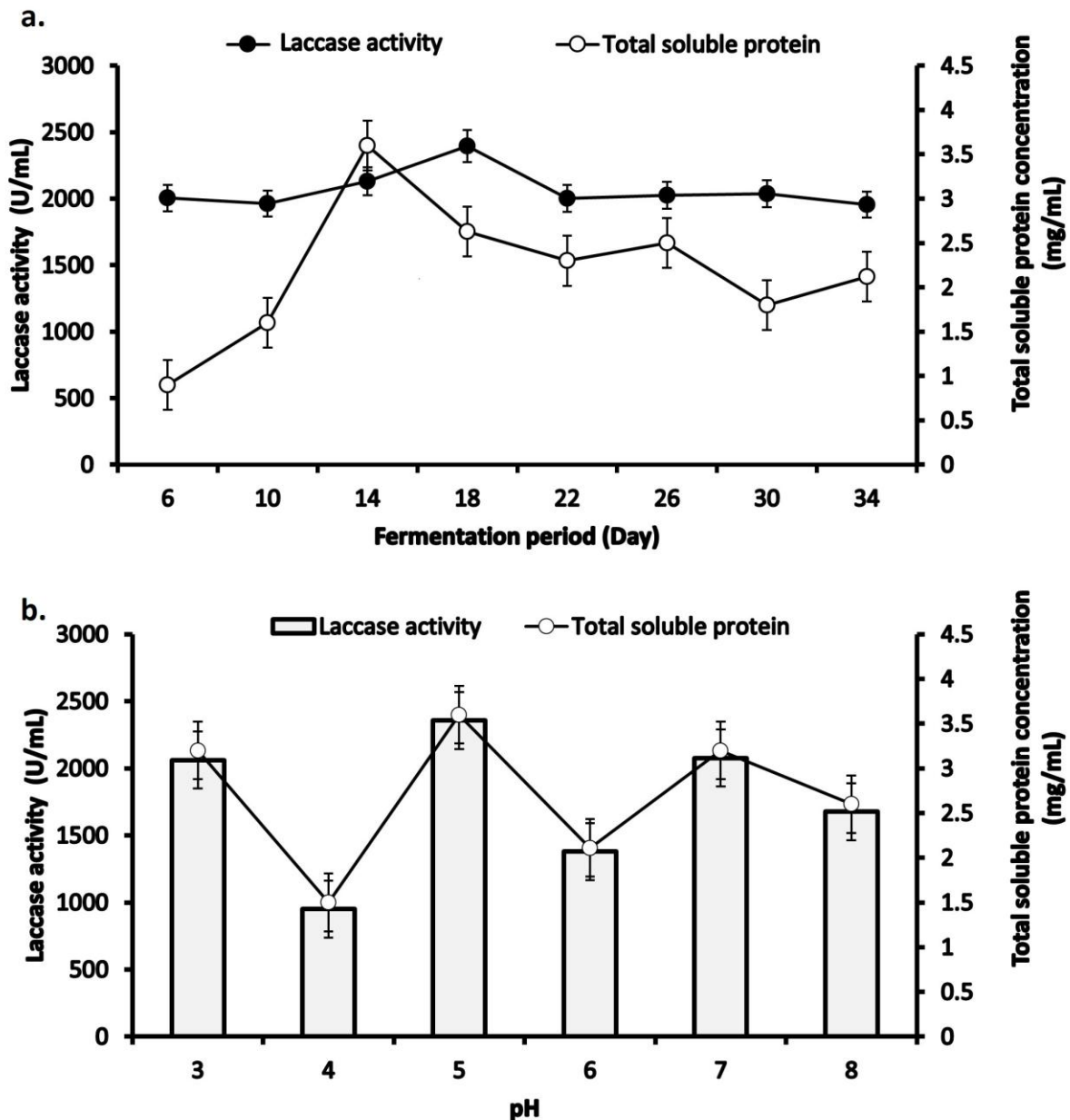
187 Results obtained from this study were subjected to analysis of variance using one way ANOVA and
188 differences between means of test samples were separated by Duncan Multiple Range Test [39].

189 3. RESULTS AND DISCUSSION

190 Fig. 1. present the fermentation period and optimum pH for laccase production by *Trametes sp.*
191 isolate B7 (GenBank accession number MK024175). The optimum fermentation period for Total
192 Soluble Protein (TSP) and laccase production were day 14 and day 18 respectively in solid state
193 fermentation of *Terminalia superba* sawdust (Fig. 1 a) while the optimum pH for TSP (3.6 mg/mL) and
194 laccase (2356 U/mL) were produced by *Trametes sp.* isolate B7 at pH 5.0 (Fig. 1 b). Many
195 investigators have reported different incubation periods for optimum production of crude laccases.
196 Some authors reported maximum laccase production on day 7 and day 10 of incubation using
197 *Lentinus edodes* and *Ganoderma sp.* respectively while another work reported maximum laccase
198 production on day 11 with rubber wood sawdust [22, 40, 41]. In another study, maximum production
199 of laccase by *Ganoderma lucidum* was obtained on day 16 which was close to our finding [42]. Some
200 authors have reported maximum production of laccase by several fungi species including *T. versicolor*

201 within the range of pH 3.5 - 7.0 which corroborates our work [41, 43]. It has been established that the
 202 optimum pH for laccase production is dependent on the species and strain in addition to the
 203 lignocellulosic substrate [44]. The laccase was 2.3 and 9.0 times purified with specific activity of 1487
 204 U/mL and 5380 U/mL for pellets and dialysate after ammonium sulphate precipitation and dialysis
 205 respectively as earlier described [27].

206



207

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

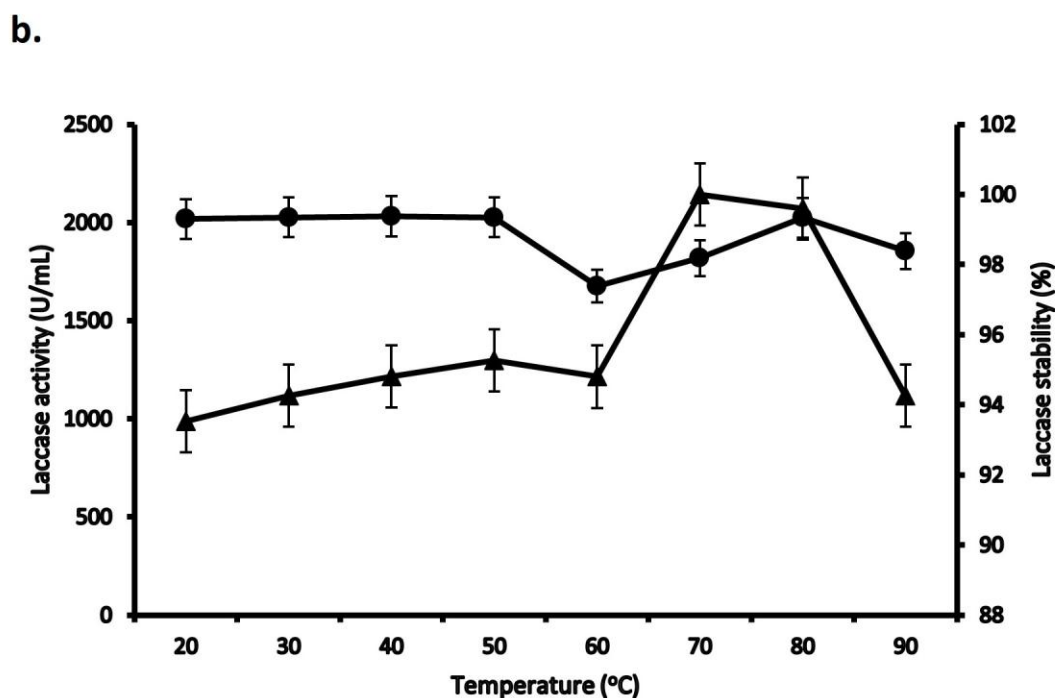
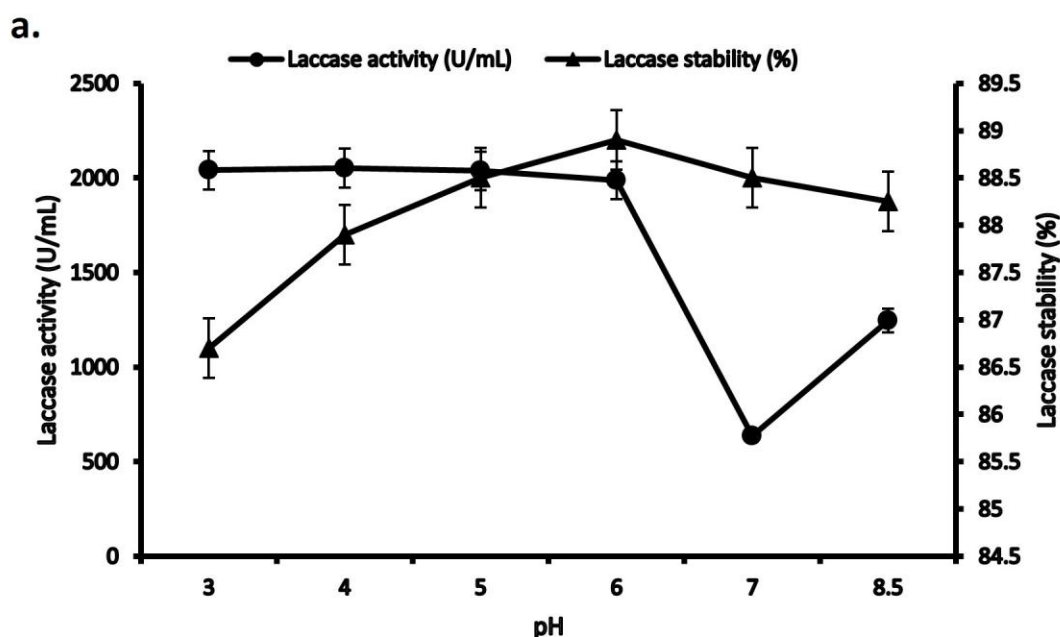
212 The activity and stability of the partially purified laccase are presented in Fig. 2a. The partially purified
 213 laccase was active in the acidic pH 3.0 - 6.0. It has been reported that most fungal laccases are active
 214 in the acidic range of pH 3.0 - 4.0 [37]. For instance, *T. versicolor* laccase has optimum activity at pH
 215 3.5 [45]. Another author reported the characterization of extracellular laccases from *Fomes annosus*,
 216 *Pleurotus ostreatus*, *Trametes versicolor*, *Rhizoctonia praticola* and *Botrytis cinerea* and observed

217 that the optimum activity varied between pH 3.0 - 5.0 which was close to this study [46]. The purified
218 laccase had high stability in a pH range of 3.0 - 8.5 and with optimum stability of 89% at pH 6.0. This
219 suggests that the enzyme may be useful in many biotechnological processes, especially those that
220 require acidic conditions. This goes in line with a report that purified laccases of basidiomycete
221 *Funalia trogii* (Berk.) Bondertsev & Singer exhibited broad pH activity and with optimum at pH 4.0
222 using 2, 6-dimethoxyphenol (DMP) as substrate [47]. These differences in peaks of optimum pH may
223 be due to differences in fungal species as well as the substrates used in characterization of the
224 enzymes.

225 The laccase was active and stable over a wide temperature range (Figure 2b). Laccase activity was
226 high in the range of 20 °C - 50 °C and 80 °C. One author reported the optimum temperature for
227 maximum activity of laccase produced by *T. versicolor* as 40 °C [45] which falls within the range of 20
228 °C - 50 °C observed in this study. The enzyme was stable from 30 °C to 80 °C with an optimum
229 stability of 100% at 70 °C. The enormous stability of this enzyme makes it more attractive for
230 biotechnological and industrial applications. Other studies indicated 30 °C - 60 °C as optimal
231 temperature range for most fungal laccases and 55 °C - 65 °C for laccases with thermophilic
232 properties similar to thermostable laccases from basidiomycetes strains [29, 48].

233

UNDER PEER REVIEW



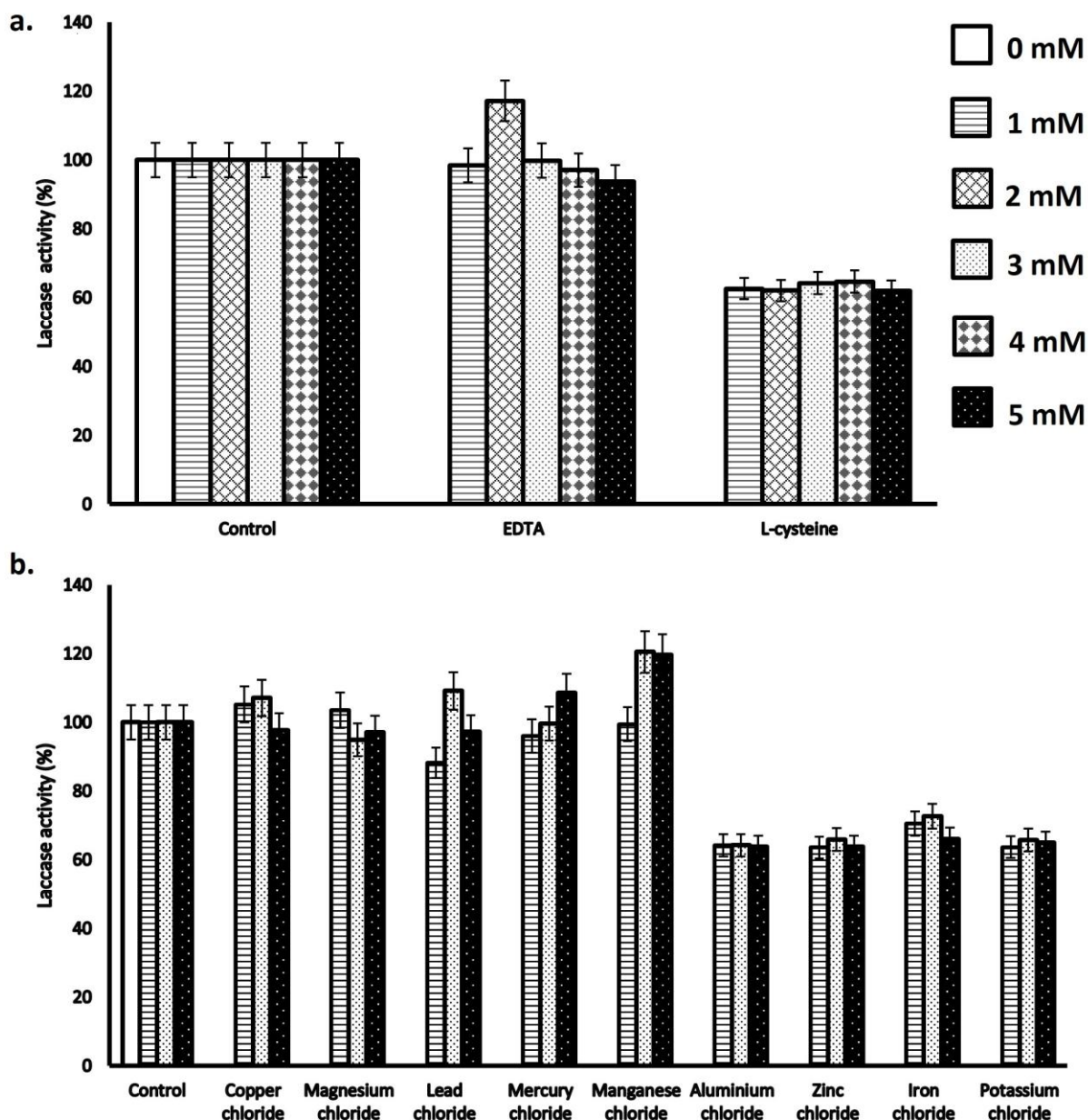
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235 Figure 2 Effect of pH (a) and temperature (b) on partially purified *Trametes sp.* isolate B7 laccase
 236 activity and stability. **Bar represent standard error of duplicate determination.**

237 EDTA was mildly inhibitory to laccase activity at 1 mM (0.55%), 3 mM (0.18%), 4 mM (2.92%) and 5
 238 mM (6.70%) but activated laccase activity at 2 mM concentration with 117%. However, L-cysteine was
 239 inhibitory to laccase activity at all the concentrations with 37.74%, 37.92%, 35.78%, 35.36% and
 240 38.10% at 1 mM, 2 mM, 3 mM, 4 mM and 5 mM respectively (Figure 3a). EDTA is an inhibitor of
 241 metallo-enzymes including laccase due to its property of forming inactive complexes with inorganic
 242 prosthetic groups/co-factors of the enzyme [17]. It has been established that L-cysteine is a stronger
 243 inhibitor of laccase activity than EDTA which agreed with our study [33]. The ability of the purified
 244 enzyme to with stand the inhibitory effects of EDTA is important for industrial and biotechnological
 245 processes which require enzymes that are resistance to metallic ions and other inhibitors especially
 246 EDTA [14].

247 Five metal ions namely Mn^{2+} , Pb^{2+} , Hg^{2+} , Cu^{2+} and Mg^{2+} activated the partially purified laccase with
 248 120.5%, 109%, 108%, 107.10% and 104% respectively (Figure 3b). The inhibitors were Al^{3+} , K^{2+} , Zn^{2+}
 249 and Fe^{2+} with inhibitions of 35.20%, 34.30%, 34.20% and 27.33% respectively (Figure 3b). In the
 250 presence of various salts, metallic ions affect enzymes substantially [37]. Report indicate that
 251 Mn^{2+} , Mg^{2+} and K^+ had high stabilizing effects on laccase from *T. versicolor* while Zn^{2+} and Cu^{2+} had
 252 destabilizing effects and in extreme cases complete loss of enzyme activity was recorded in the
 253 presence of Cu^{2+} and Fe^{2+} [37]. Another work reported higher inhibitory rates of 64% and 55% for Zn^{2+}
 254 and K^+ respectively for purified laccase of *Lentinula edodes* [17]. This variation is because the effect
 255 of metal ions on laccase activity is highly dependent on its source and the type of metals used, which
 256 have a great influence on the catalytic activity of the enzyme [17].

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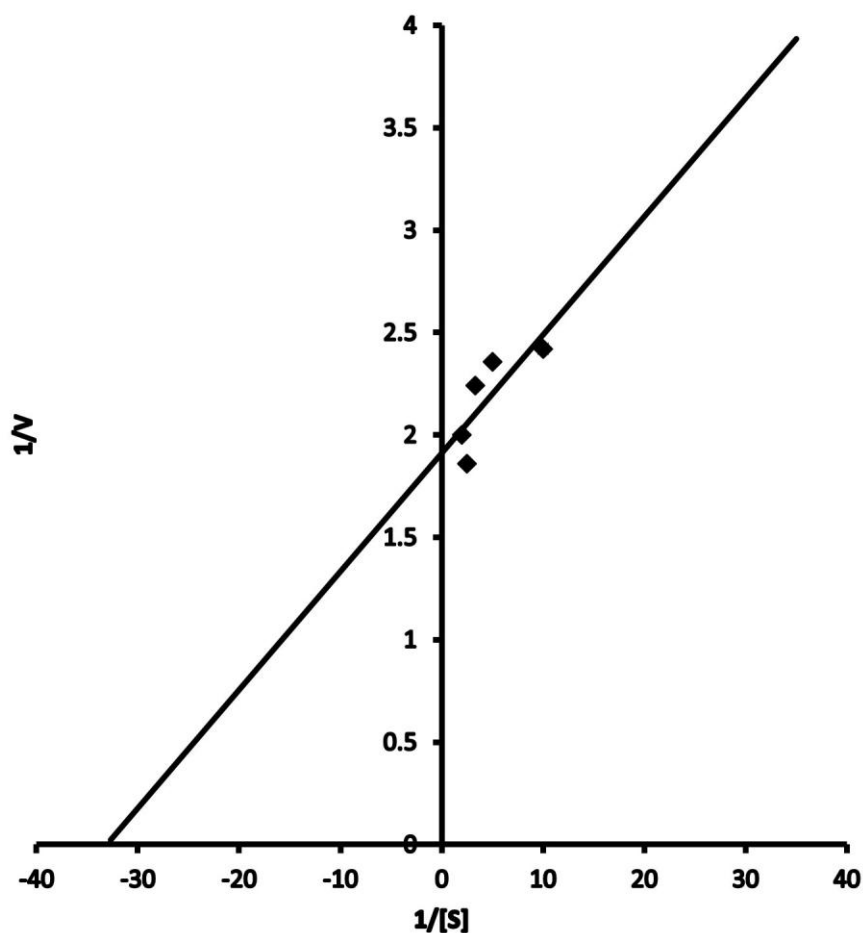


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259 Figure 3 Effect of (a) EDTA, L-cysteine and (b) metal ions concentration on the activity of partially
 260 purified *Trametes sp.* isolate B7 laccase. Bar represent standard error of duplicate determination.

261 The purified laccase had K_M 33 μM and V_{max} 1.91 $\mu Mol./min/mL$ (Figure 4). The authors of one study
 262 reported K_M 180 μM and V_{max} 3.95 $\mu mol/min/mg$ for purified laccase from *T. harzianum* [17] which

263 implied that the partially purified laccase of *Trametes sp.* isolate B7 had a higher substrate affinity
264 than that of *T. harzianum*. This is because the rate of reaction and concentration of substrate depends
265 on its K_M and the lower the K_M the higher the substrate affinity [34]. A study to determine the
266 molecular weight of the purified laccase showed a monomeric enzyme with molecular weight of ~36
267 kDa using one-dimensional native gel electrophoresis as described [27]. The molecular weight was
268 lower than other laccases which were reported in the range of 55 - 65 kDa, however; it was higher
269 than that of *L. polychrous* with about 32 kDa [49]. Another study detected the presence of two iso-
270 laccases with one of the bands at 38 kDa which is close in molecular weight to that of *Trametes sp.*
271 isolate B7 laccase [50].



272

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

275 4. CONCLUSION

276 The study established *Terminalia superba* sawdust as an alternative low-cost substrate for production
277 of cheap laccases utilizing *Trametes sp.* isolate B7. Production of the laccase was optimum at pH 5.0
278 on day 18. Characterization of laccase showed high enzyme activity in acidic pH and a broad enzyme
279 stability in acidic to mild alkaline range. In addition it was active at high temperature, thermostable at
280 70 °C for 1 hr and resistant to most metallic ions and EDTA. These attributes made the enzyme a
281 potential tool for many biotechnological and industrial applications including those of pulp and mill
282 paper, polycyclic aromatic hydrocarbons, pesticides and dyes wastewaters among other xenobiotics.

283 Ethical declaration and consent not applicable.

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