Original Research Article

Screening, Production and Partial Characterization of a Thermostable Laccase from *Trametes* sp. Isolate B7 with Biotechnological potentials.

ABSTRACT

The search for efficient and green oxidation technologies has increased interest in utilization of laccases in non conventional methods. Laccases catalyze a wide range of substrates due to low substrate specificity and strong oxidative potentials. Challenges to large-scale enzyme utilization include, low enzyme activity and instability which restrict use in many areas of biotechnology. In the study, 59 fungi comprising *Aspergillus niger* (40%), *Trichoderma harzianum* (31%), *Aspergillus flavus* (9.0%), *Trichoderma viride* (5.0%), *Fusarium oxysporum* (5.0%), *Rhizopus stolonifer* (5.0%), *Trametes* sp. (3.0%) and *Aspergillus nidulans* (2.0%) were isolated and screened for laccase production. Plate screening test showed 57.5%, 34.0% and 8.5% of fungi were laccase-positive on ABTS, Guaiacol, and α-naphthol agar respectively. Isolates were further screened in liquid cultures, and the highest laccase producer identified molecularly. *Trametes* sp isolate B7 was selected for solid state fermentation (SSF). Laccase production in SSF was highest at pH 5.0 (2356 U/mL). The purified laccase showed high activity (pH 3.0 - 6.0) and stability (pH 3.0 - 8.5) using ABTS. It was active (20 - 80^{o}C) and thermostable (30 - 80^{o}C) with optimum stability at 70^{o}C (100% for 1 hour). The percentage decolourization of Phenol red were 28% and 36% using 1000 U/mL and 2000 U/mL crude laccases respectively. Similarly, RBBR (100%), Congo red (75%) and Malachite green (62%), and 77.4%, 64% and 28% were decolourized using 1000 U/mL and 2000 U/mL crude laccases respectively. ABTS agar was very reliable in large-scale screening for laccase which possessed thermostable property and degraded synthetic dyes without use of enzyme mediators. These attribute made the enzyme suitable for application in industry and biotechnology.

Keywords: Trametes sp. B7, Thermostable laccase, ABTS agar, Guaiacol agar, α-naphthol agar, Synthetic dyes, Solid State Fermentation.

1. INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductase; E.C.1.10.32.) are multi-copper extracellular glycoproteins with molecular weight of 60 - 80 kDa having 15 - 20% carbohydrate content and are wide spread in bacteria, insects, lichen and plants apart from fungi of diverse eco-physiological groups [1, 2]. Laccases possess strong oxidation potentials that allow catalysis of a broad range of substrates such as poly-phenols, substituted phenols, diamines and some inorganic compounds [3]. The oxidation reaction is coupled to the reduction of molecular oxygen with a one-electron mechanism. They contain four copper atoms in one molecule and the coppers belong to three different types, which can be distinguished using UV/visible spectroscopy and electron paramagnetic resonance spectra [4].

For extracellular production of proteins, fungi species have several advantages over bacteria because they are characterized by high-level secretion of enzymes during decomposition [5]. In culture, laccases are usually the first ligninolytic enzymes secreted to the surrounding media by fungi [6]. The majority of isolated and characterized laccases are of fungal origin. These include *Agaricus, Trametes* (syn. *Polyporus,* syn. *Coriolus*), *Pleurotus, Podospora, Rhizonia, Neurospora, Aspergillus, Phlebia, Botrytis, Cerrena and Myceliophthora* [3]. Fungal laccases are more useful in industrial and biotechnological applications due to their catalytic properties, high redox potentials and low substrate specificity for synthetic dyes and many other xenobiotics [7].

Laccases catalyze the oxidation of organic compounds in the absence of H_2O_2 or Mn^{2+} which allow their application in several industrial processes [8]. They often face harsh conditions in industrial processes, such as high temperature, high salt concentration, and extremely acidic or alkaline pH [9]. Low yield and enzyme instability are important factors preventing large-scale application of laccases in biotechnology [10]. To this end, discovery of novel laccases with high yield and improved stabilities is necessary for industrial utilizations.

Screening of fungal laccases has been carried out either on solid media or in liquid cultures [3]. Solid screening using coloured indicators is simpler and does not involve sample handling or measurements. Studies have reported the use of ABTS, guaiacol and *α*-naphthol in plate screening for visual identification of laccases [8]. Oxidation of guaiacol presents reddish brown colour, *α*-naphthol a deep purple colour, and 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) a dark green halo around laccase-forming colonies on agar [11]. Most fungal laccases prefer ABTS as the ideal substrate [8]. However, reports indicate the ability of peroxidises to oxidize ABTS in the presence of H_2O_2 which might be produced endogenously [12]. Consequently, Azure B is use to confirm the absence of peroxidises [12]. The use of different indicators for laccase screening allows comparison and could reduce the number of indicator compounds required in future.

The pH of a culture medium plays critical role in growth and production of laccases by organisms. One author reported maximum production of fungal laccases at pH 5.0 [3]. Most reports have indicated pH levels of 4.0 - pH 6.0 as optimum for fungal laccases especially in solid state fermentation [3]. The optimum temperature for laccase production differs greatly from one strain to another. When incubated in light, production of fungal laccases is optimum at 25^{o}C whereas optimum production in the dark is at 30^{o}C [13]. The thermo-stability of a laccase varies considerably with the source of organism. Generally, laccases are stable at 30 - 50^{o}C and rapidly lose activity at temperatures above 60^{o}C [14]. The majority of fungal laccases operate in the range of 30 - 55℃, and their optimum pH range is limited to mildly acidic conditions. Thus, identification of laccases that are robust to harsh conditions in biotechnology could improve economic viability of this process.

The search for efficient and green oxidation technologies has increased interest in use of enzymes to replace conventional non biological methods [15]. Laccases as "Green Tools" require only molecular oxygen for bio-catalysis and not hydrogen peroxide. Due to their high catalysis they are utilized in bio-synthesis, energy exploitation, environmental protection, bio-detection and degradation of synthetic dyes among others [16]. More than 100,000 dyes are commercially available with annual production of over 7×105 tonnes [17]. Extensive utilization of these are reported in diverse areas of industry with approximately 10 - 15% of spent dyes discharged as wastewater in to the environment [18]. Most synthetic dyes are highly stable in presence of light and high temperatures, and are fast to detergents and resistant to microbial attacks. Furthermore, they exist in wide arrays of colours, and are easy and cheap to synthesize when compared to natural dyes [19].

However, many synthetic dyes are toxic, carcinogenic or prepared from known carcinogens or other aromatic compounds that may cause harm to humans, animals or other forms of aquatic resources [20]. Several studies have reported the decolourization of synthetic dyes by fungal species using laccase as the main enzyme during the process [20]. The use of laccase in biological treatment effectively oxidizes pollutants to less soluble compounds that are easily removed by sedimentation and filtration [21] In addition, biological treatments are less expensive and environmentally-friendly.

Though laccases exhibit great industrial and biotechnological potentials limitations like instability in varying pH, low tolerance to high temperatures and purification processes restrict its application. Therefore, identification of thermo-stable laccases and utilization of cell-free extracts would present enzymes that possess high catalysis, high substrate specificity, shorter reaction time and mild reaction conditions in the industrial sector. The objective of this study is to isolate and screen laccase-producing fungi from wood samples using enzyme indicators and identify extracellular laccases with industrial and biotechnological potentials.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Fungal Strain

The fungi used in this study were isolated from sawdust dump sites in Gboko and Makurdi plank markets, and decaying woods in Benue Polytechnic Campus, Ugbokolo, Benue State, Nigeria. Pieces of samples were placed on sterile Potato Dextrose Agar (PDA) plates and incubated at 27 ± 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 [22].

2.2 Screening of Fungal Strain for Laccase Production

2.2.1 Primary screening of fungal strain on solid media

The Lignin Basal Medium (LBM) used for primary screening consisted of the following composition (gL^{-1}) in distilled water. KH_2PO_4 1 g, $C_4H_{12}N_2O_6$ 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $CaCl_2.2H_2O$ 0.01 g, Yeast extract 0.01 g, $CuSO_4.5H_2O$ 0.001 g, $Fe_2(SO_4)_3$ 0.001 g, $MnSO_4.H_2O$ 0.001 g [23]. The LBM medium was separately supplemented by incorporating 0.1% (w/v) ABTS, 0.01% (w/v) guaiacol, 0.005% (w/v) α-napthol and 0.01% (w/v) Azure B with 1.6% (w/v) agar-agar and autoclaved at 121^{o}C (15 psi) for 15 minutes. Twenty percent (w/v) aqueous glucose solution was separately sterilized at 110^{o}C (10 psi) for 10 minutes and 1 mL of this added to each 100 mL of the sterilized medium [24]. The medium was

aseptically transferred into sterile petri dishes (60 mm in diameter) and inoculated with 5 mm agar disk of active fungal mycelia from 5-day old culture. Isolates were incubated at 27 ± 2 °C for 10 days in darkness. Colonies with dark green halo on ABTS agar and with a diameter above 1 cm were considered highly ligninolytic [25, 26] while those with a dark brown or puplish halos on guaiacol and α-napthol agar respectively were positive for laccase activity [24] and selected for secondary screening.

2.2.2 Secondary screening of fungal strain in liquid medium

Fungal isolates were subjected to quantitative determination of laccase activity in 500 mL baffled Erlenmeyer flasks (with rotary shaking 3 RCF for 20 minutes) containing 50 mL Lignin Modifying Medium (LMM) with 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 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 [7]. The LMM was autoclaved at 121°C (15 psi) for 15 minutes while 1% (w/v) aqueous glucose solution was separately autoclaved at 110°C (10 psi) for 10 minutes and 1 mL added to each 100 mL of the medium. Two agar disks (5 mm diameter) of active fungi mycelia were inoculated and incubation carried out at 27 ± 2°C for 3, 6, 9 and 12 days.

2.3 Assay of Laccase from Secondary Screening

Laccase activity was determined by following the oxidation of ABTS at 420 nm using spectrophotometer. The reaction mixture consisted of 600 µL sodium acetate buffer (0.1 M, pH 5.0 at 27℃), 300 µL 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ABTS (5 mM), 300 µL culture supernatant and 1400 µL distilled water. The reaction was incubated for 2 minutes at 30℃ and initiated by adding 300 µL hydrogen peroxide and absorbance measured after one minute [27]. One Unit of laccase activity was defined as the activity of an enzyme that catalyzes the conversion of 1 µmol of ABTS (ε=36,000 M-1 cm-1) per minute.

2.4 Production of Laccase in Solid State Fermentation (SSF)

2.4.1 Selection of fungal isolate for inoculation

The fungal isolate with the highest production of laccase during secondary screening was subjected to molecular and phylogenetic characterization, and identified as *Trametes* sp. isolate B7. The sequence data was deposited in GenBank under the accession number MK024175 [28]. Based on the quantitative assay the fungus was selected for inoculation in SSF.

2.4.2 Substrate collection and processing

Wood samples of *Terminalia superba* used in the study were collected from Gboko plank market, Benue State, Nigeria and processed into sawdust as earlier described [29].

2.4.3 Medium and culture conditions

The Lignin Modifying Medium (LMM) used to moisten the sawdust sample was adjusted to pH 5.0 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 [7]. 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°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. 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 ± 2°C for 18 days [30].

2.5 Extraction and Partial Purification of Crude Laccase

The extracellular enzymes were extracted by adding 100 mL of 0.1M citrate-phosphate buffer (pH 5.0) into the fermenting flask. The mixture was stirred for 30 minutes with a glass rod 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. The extract was centrifuged at 17150 RCF for 20 min, at 4°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 [31]. 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 [31]. Enzyme activity was determined before and after dialysis.

2.6 Partial Characterization of Laccase

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

2.6.1 Effect of pH on laccase activity

The effect of varying pH on laccase activity was carried out using different buffers (600 µL), adjusted to pH values ranging from 3.0 - 8.5, 300 µL ABTS (5 mM), 300µL culture supernatant 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 [27]. 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 [32].

2.6.2 Effect of pH on laccase stability

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°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 [30, 33].

2.6.3 Effect of temperature on laccase activity

The effect of varying temperature on laccase activity was carried out at different temperatures from 20 - 90°C at pH 5.0. The enzyme was incubated for 15 minutes and assayed by standard protocol [32].

2.6.4 Effect of temperature on Laccase thermo-stability

The stability of enzyme under different temperature was evaluated by using 0.1 M sodium acetate (pH 5.0), and incubating at 20 - 90°C for 1 hour. A 300 µL aliquot of the enzyme was withdrawn and placed on ice before assaying for remaining enzyme activity using standard assay protocol [30, 33].

2.7 Dye decolourization potential of crude laccase

The decolourizing potential of crude laccase was tested using selected dyes at concentration of 0.01% (w/v) dissolved in sterile distilled water. The reaction mixture consisting of equal volume aqueous solution of dye and crude protein of 1000 U/mL and 2000 U/mL (1:1) separately in citrate phosphate buffer (pH 5.0) was incubated at 27 ± 2°C in the dark for 1, 24, 48, 72 and 120 hours. Decolourization of dyes was determined by monitoring the decrease in absorbance at the wavelength of maximum absorption for each dye: Phenol Red (475 nm), Congo Red (497 nm), Crystal Violet (590 nm), Remazol Brilliant Blue Royal (RBBR) (587 nm) and Malachite Green (620 nm) [21, 34]. Control tests were performed using a heat-denatured crude enzyme. The experiment was carried out in triplicates and activity of decolourization calculated thus:

$$D\% = 100 \times \frac{(A_{ini} - Af_{in})}{A_{ini}}$$

Where,

D = Decolourization.

A_{ini} = Initial absorbance.

A_{fin} = Final absorbance of dye after incubation time.

[21].

2.8 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 [35].

3. RESULTS AND DISCUSSION

Screenings for laccase production have been primarily carried out in wood rot fungi species of the family basidiomycetes. However, recent studies have explored screenings for laccases in other fungal families. In this study, a total of 59 fungal strains were isolated, identified and screened for laccase production among which were members of the fungal family *Ascomycetes*. The isolated fungi comprised of *Aspergillus niger* (40%), *Trichoderma harzianum* (31%), *Aspergillus flavus* (9.0%), *Trichoderma viride* (5.0%), *Fusarium oxysporum* (5.0%), *Rhizopus stolonifer* (5.0%), *Trametes* sp. (3.0%) and *Aspergillus nidulans* (2.0%). Similar screening for laccase production by *Aspergillus niger*, *Aspergillus flavus*, *Trichoderma harzianum*, *Trichoderma viride*, *Fusarium* sp. among others has been reported using guaiacol as indicator [36]. Figure 1 present the percentage frequency of occurrence of the fungal isolates on PDA. The diversity and spread of these fungal species probably reflected their unique ability to degrade some components of the wood due to capability to synthesize the relevant hydrolytic and oxidative extracellular enzymes necessary for mineralization of the lignocellulosic substrates [7].

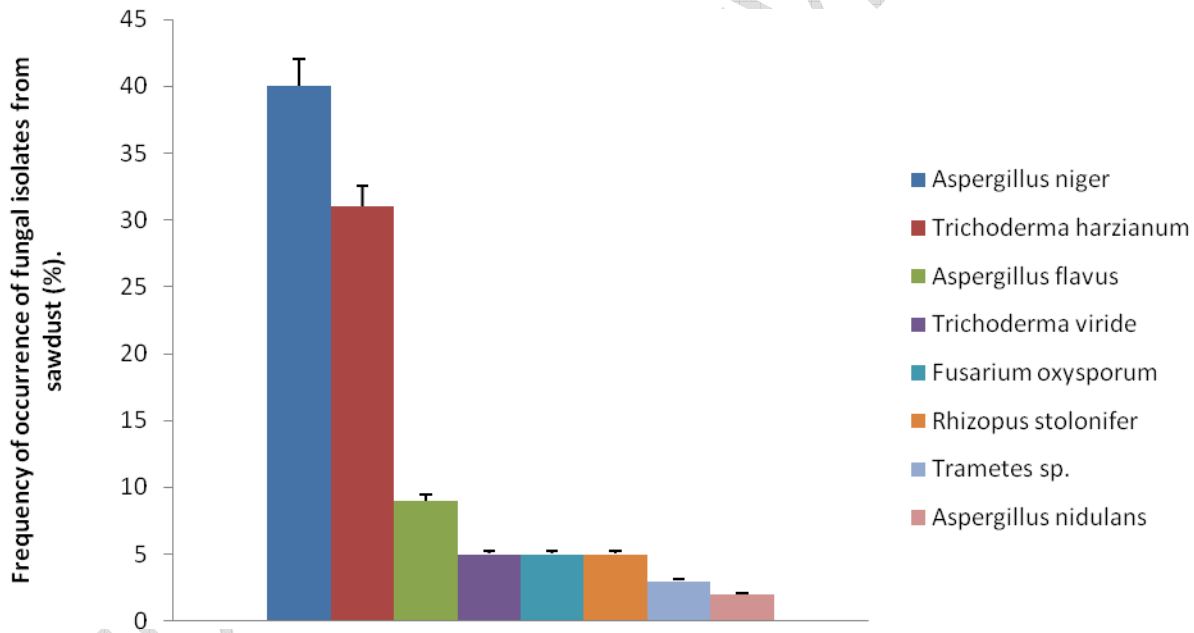

Figure 1 Percentage frequency of occurrence of fungal isolates from sawdust samples of *Terminalia superba.* Bar represent standard error of the mean.

Table 1 shows the qualitative screening of fungal isolates for laccase activity using ABTS, Guaiacol and α-napthol agar. Results showed that 57.5% were laccase-positive on ABTS agar while 34.0% and 8.5% were laccase-positive on α-napthol agar and guaiacol agar respectively. The result of screening with three different chromogenic indicators showed no correlation as majority of laccase-positive isolates on ABTS agar failed to demonstrate similar sensitivity on guaiacol agar and α-napthol agar. This agreed with the findings of another study which reported that most fungal laccases prefer ABTS as the ideal substrate [8]. Furthermore, laccases from different organisms exhibit considerable diversity in substrate specificity as well as other properties [14, 37]. The use of ABTS as substrate for screening of laccase provides rapid visualization and confirmation of the enzyme. However,

report indicates the ability of peroxidase enzymes to also oxidize ABTS in the presence of H_2O_2 which might be produced endogenously [12]. To rule out the possibility of false-positive results for laccase production on ABTS agar; all isolates were plated on Azure B agar and confirmed negative for peroxidase enzyme [12]. Therefore, the study established ABTS as a straight forward, rapid, reliable and visual substrate for large-scale plate screening of laccases which is devoid of sample handling and measurements [38].

However, in another study, out of 25 fungal isolates screened for laccase production, 3 isolates were laccase-positive on Tannic acid agar, 2 isolates on guaiacol agar while ABTS agar recorded 1 laccase-positive isolate [39]. In another study, *Aspergillus niger*, *Tichoderma* harzianum, *Trichoderma viride* and *Fusarium solani* were screened for laccase activity using guaiacol, and only *Fusarium solani* was positive for laccase production [36]. In this study, *Aspergillus niger,* and *Trichoderma harzianum* were laccase-negative on guaiacol agar but tested positive on ABTS agar along with *Aspergillus nidulans*. Interestingly, three isolates in the study, namely; *Trametes* sp. isolate B7 MK024175, *F. oxysporum* B34 and *Trametes* sp. G31 showed a strong correlation on all the three indicators by testing laccase-posivitive.

315 **Table 1 Qualitative screening of fungal strains on solid media incorporated**
316 **with different indicator compounds**
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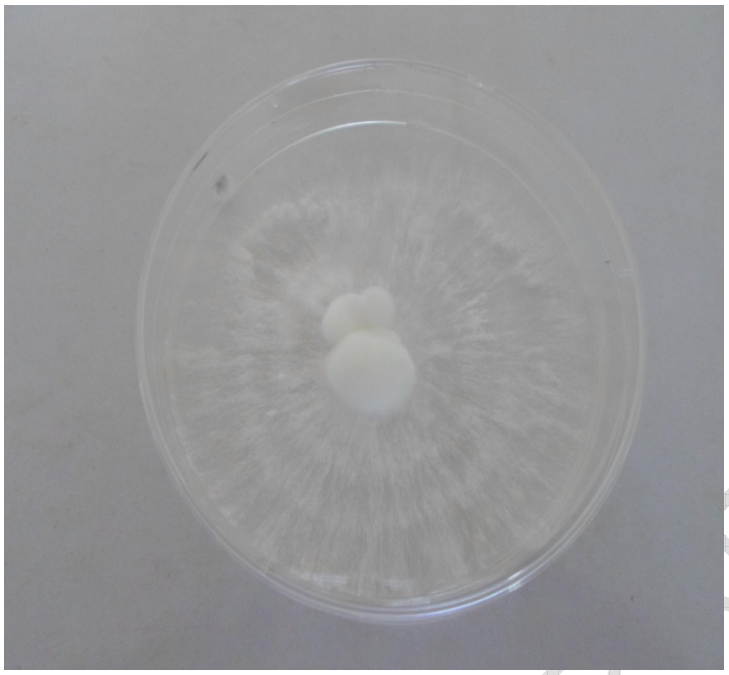
S/No.	Code	Fungi	ABTS	Guaiacol	α-Napthol	Azure B
1	B2	*A. niger*	+	-	-	-
2	B4	*F. oxysporum*	+	-	+	-
3	B5	*T. viride*	-	-	+	-
4	B6	*A. niger*	-	-	-	-
5	B7	*Trametes* sp.B7	+	+	+	-
6	B9	*A. niger*	-	-	-	-
7	B10	*A. niger*	-	-	-	-
8	B12	*T. harzianum*	-	-	-	-
9	B13	*A. niger*	-	-	+	-
10	B14	*A. niger*	-	-	+	-
11	B15	*A. niger*	+	-	-	-
12	B16	*A. niger*	+	-	-	-
13	B21	*A. flavus*	-	-	-	-
14	B22	*A. niger*	-	-	-	-
15	B28	*A. niger*	+	-	-	-
16	B34	*F. oxysporum*	+	+	+	-
17	G1	*T. harzianum*	-	-	-	-
18	G2	*T. harzianum*	-	-	+	-
19	G3	*T. viride*	-	-	-	-
20	G4	*A. niger*	+	-	-	-
21	G5	*T. viride*	-	-	-	-
22	G6	*A. flavus*	-	-	-	-
23	G7	*T. harzianum*	-	-	+	-
24	G8	*T. harzianum*	+	-	-	-
25	G9	*T. harzianum*	-	-	-	-
26	G10	*R. stolonifer*	-	-	-	-
27	G11	*T. harzianum*	-	-	+	-
28	G15	*T. harzianum*	-	-	-	-
29	G17	*A. niger*	+	-	-	-
30	G18	*A. niger*	+	-	-	-
31	G26	*T. harzianum*	+	-	-	-
32	G29	*A. flavus*	-	-	-	-
33	G31	*Trametes.* sp	+	+	+	-
34	G32	*T. harzianum*	-	-	+	-
35	G33	*T. harzianum*	-	-	-	-
36	G34	*F. oxysporum*	+	-	-	-
37	G37	*R. stolonifer*	-	-	-	-
38	G38	*T. harzianum*	-	-	-	-
39	M2	*A .niger*	+	-	-	-
40	M6	*T. harzianum*	-	-	+	-
40	M6	*T. harzianum*	-	-	+	-
41	M8	*A. niger*	+	-	-	-
42	M10	*R. stolonifer*	-	+	+	-
43	M11	*T. harzianum*	+	-	-	-
44	M12	*T. harzianum*	-	-	-	-
45	M13	*A. flavus*	-	-	-	-
46	M14	*A. niger*	+	-	+	-
47	M17	*T. harzianum*	-	-	+	-

S/No.	Code	Fungi	ABTS	Guaiacol	α-Napthol	Azure B
48	M18	*A. niger*	+	-	+	-
49	M19	*A. niger*	+	-	-	-
50	M20	*A. niger*	+	-	-	-
51	M22	*A. niger*	+	-	-	-
52	M23	*A. niger*	+	-	-	-
53	M24	*A. flavus*	-	-	-	-
54	M25	*T. harzianum*	-	-	-	-
55	M26	*T. harzianum*	-	-	-	-
56	M27	*A. niger*	+	-	-	-
57	M28	*A. niger*	+	-	-	-
58	M29	*A. nidulans*	+	-	-	-
59	M31	*A. niger*	+	-	-	-

Plate 1 (a) shows cultural characteristic of *Trametes* sp. B7 on PDA plate, (b) presents *Trametes* sp. B7 on ABTS agar showing a dark green halo around the colony indicative of laccase production while (c) and (d) present *Trametes* sp. B7 on guaiacol agar and α-napthol agar with dark brown and deep purplish colours around the colonies indicating laccase production. Several authors have reported the oxidation of ABTS, guaiacol and α-napthol by laccase-producing fungi to produce dark green, reddish brown and deep purplish halos respectively, around colonies on solid media incorporated with the indicators [11, 38, 40]. Isolates which did not show any colour change lacked laccase activity and were not considered for further work. In a study, initial screening for laccase activity reported complete oxidation of ABTS and guaiacol after 7 days of incubation using *Alternaria arborescence, Aspergillus niger, Fusarium oxysporum,* and *Penicillium marneffei* [16, 40]. Another study using *P. ostreatus, Fusarum solani, Pleurotus platypus, Agaricus bisporus* and *Penicillium chrysogenum* showed oxidation of guaiacol on the 4th day of incubation [36]. However, screening of *Trametes* sp. B7 among others for laccase activity showed oxidation of ABTS, guaiacol and α-napthol right from day two of incubation. The qualitative test facilitated rapid screening of a large sample of fungal strains and revealed that 36 strains were laccase-producing. This agreed with earlier studies that laccase is more common and is usually the first ligninolytic enzyme secreted to the surrounding media by fungi in studied conditions [6].

a.



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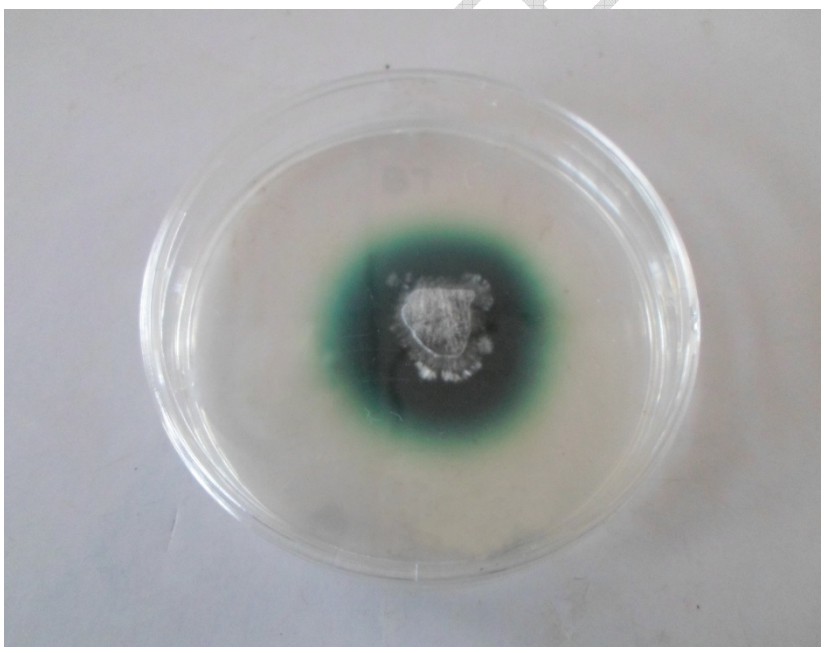

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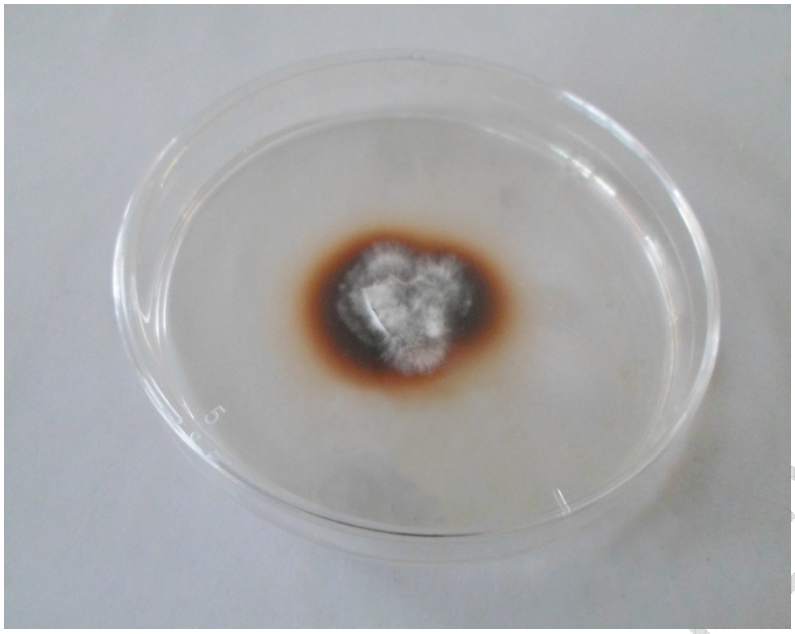

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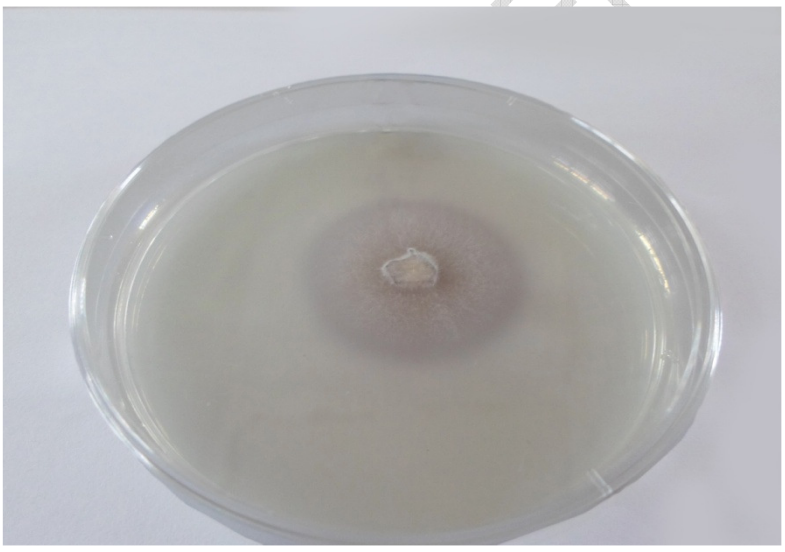

Plate 1a - d shows primary screening of fungal isolate on solid media. (a)*Trametes* sp. isolate B7 on PDA. (b) *Trametes* sp. isolate B7 on ABTS agar showing dark green halo around the colony indicating laccase production. (c) *Trametes* sp. isolate B7 on guaiacol agar showing dark brown halo around the colony indicating laccase production. (d) *Trametes* sp. isolate B7 on α-napthol agar showing deep purplish halo around the colony indicating laccase production.

Figure 2 present results of quantitative screening of laccase-producing fungi in liquid medium. Isolates varied in laccase activities during secondary screening. The highest laccase activity of 1839 U/mL was produced by *Trametes* sp. B7 on day 6. However, the enzyme activity declined as incubation progressed to day 9 and day 12. In another study, maximum laccase activity was also obtained on the 6th day of incubation which agreed with this work [41]. However, six fungal isolates were quantitatively screened by submerged fermentation in another work and the findings were different. The highest laccase activity was observed on day 3 by most isolates, and day 2 for another isolate; nevertheless, maximum activity was lower on day 2 [38]. One author reported production of extracellular laccases in log phase during secondary screening in liquid cultures [41]. Previously, activities of laccases and other ligninolytic enzyme were related to limiting nutrients in the stationary phase of growth in different fungi [42]. It has been established that the actual phase for maximum laccase activity depends on the cultivation medium [43]. The highest producer of laccase during secondary screening, *Trametes* sp. isolate B7 MK024175 was selected as starter for laccase production in SSF. Plate 2 present secondary screening of *Trametes* sp. B7 among others in liquid culture.

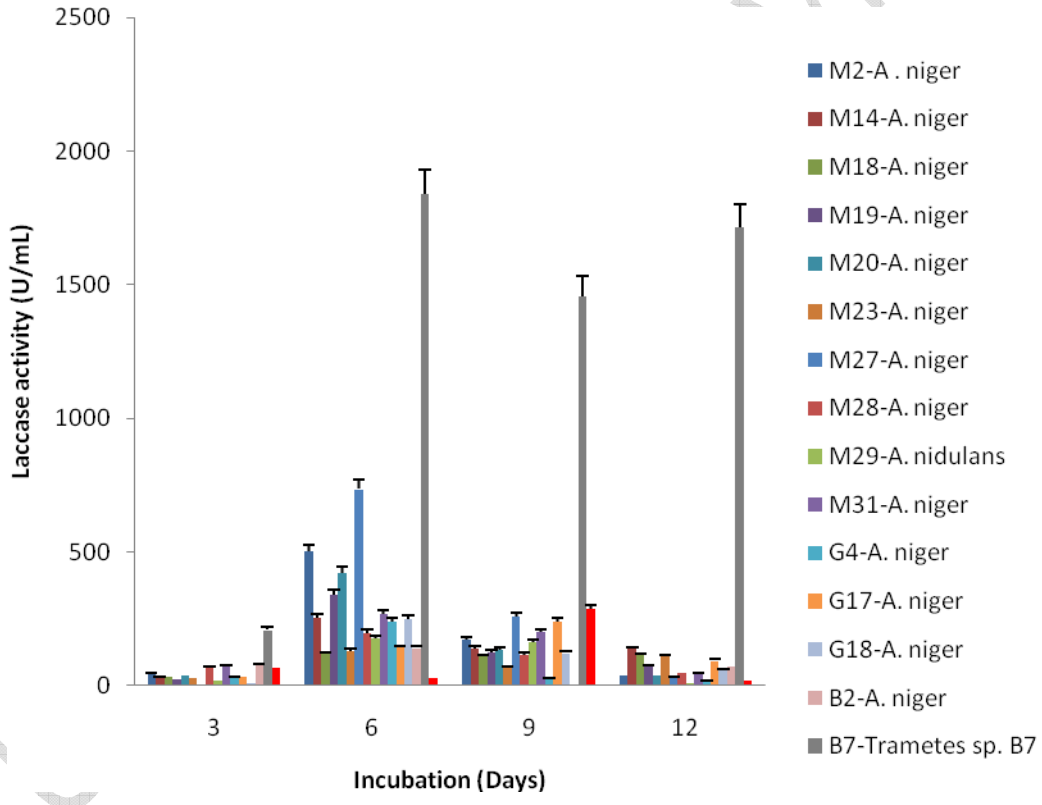

Figure 2. Quantitative screening of fungal strains for laccase production in liquid medium. Bar represent standard error of duplicate determination.

Plate 2. Showing secondary screening of *Trametes* sp. isolate B7 among others for laccase activity on the 12th day.

The pH of culture medium plays critical role in growth and production of fungal laccases. In the study, *Trametes* sp. B7 recorded maximum laccase activity of 2356 U/mL at pH 5.0 (Fig. 3). It has been reported that intial pH of 4.0 - 6.0 is optimum for production of most fungal laccases in SSF prior to inoculation [3]. Another author reported maximum production of fungal laccases when the initial pH of the growth medium was set at pH 5.0 [3] which agreed with our study. It is a fact that the optimum pH for enzyme production is dependent on the species and strain in addition to the lignocellulosic substrate [44].

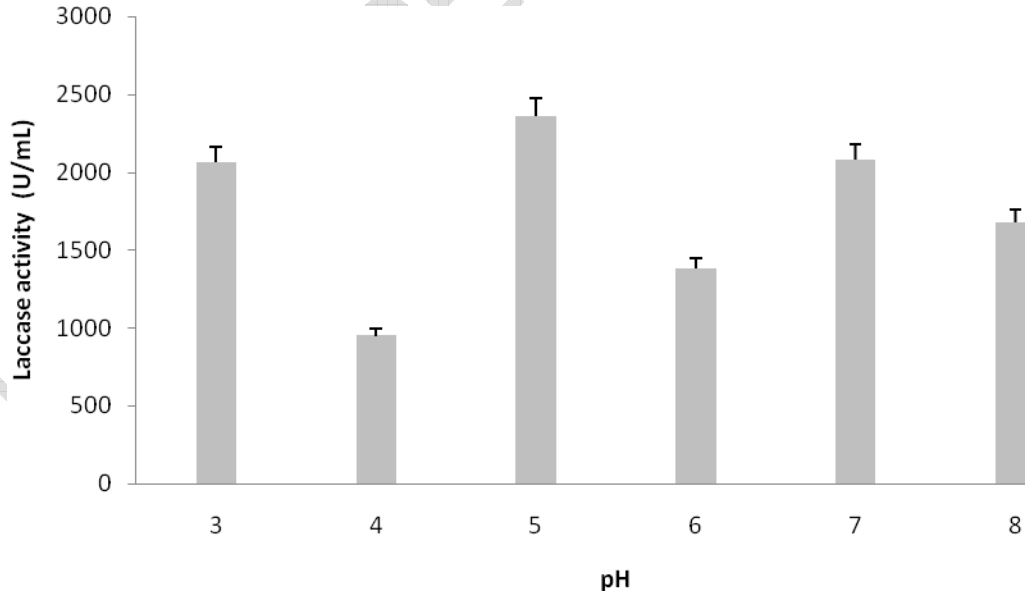

Figure 3 Effect of pH variation on production of laccase. Bar represent standard error of duplicate determination.

Laccase activity and stability are crucial for their applications in various areas of industry and biotechnology. The study investigated effect of pH variation on activity and stability of the partially purified laccase as shown in Fig. 4a. Characterization of the purified laccase showed high activity in the acidic pH 3.0 - 6.0. Many reports show that the optimum pH for laccase varies when different substrates are used; however, using ABTS as a substrate, many laccases exhibit an optimal catalytic pH value in the acidic range. [8]. Also, another study reported that most fungal laccases have pH optima in acidic range using ABTS as substrate [9, 45]. Similarly, partial characterization of purified laccase of *Cladosporium cladosporioides* showed a wide pH optima of 3.0 - 6.0 using ABTS which is consistent with the study [46]. One study 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 falls within the range of the study [47]. In addition, characterization of laccase activity from three strains of *Klebsiella pneumoniae* found pH 5.0 as optimum using ABTS [48]. The purified laccase of *Trametes* sp. B7 exhibited 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 possessed high potentials for 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 exhibit broad pH activity and optimum at pH 4.0 using 2, 6-dimethoxyphenol (DMP) as substrate [49]. The difference in pH optima was due to the fungal species and substrates used in characterization.

Furthermore, the laccase was active and stable over a wide range of temperatures (Fig. 4b). Laccase activity was high in the range of 20 - 50℃ and 80℃. One study reported maximum laccase activity of *T. versicolor* at 40℃ [50] which falls within the range of 20 - 50℃ observed in this study. The enzyme was thermostable at 30 - 80℃ and optimum stability was 100% at 70℃ for 1 hour. A similar study showed high thermal stability of *K. pneumoniae* laccases which could withstand temperatures upto 70^{o}C [48]. However, laccases of *Cladosporium cladosporioides* were stable from 40 - 70℃ but with optimum at 40^{o}C [46]. The enormous thermostability of *Trametes* sp. B7 laccase makes it more attractive for biotechnological and industrial applications.

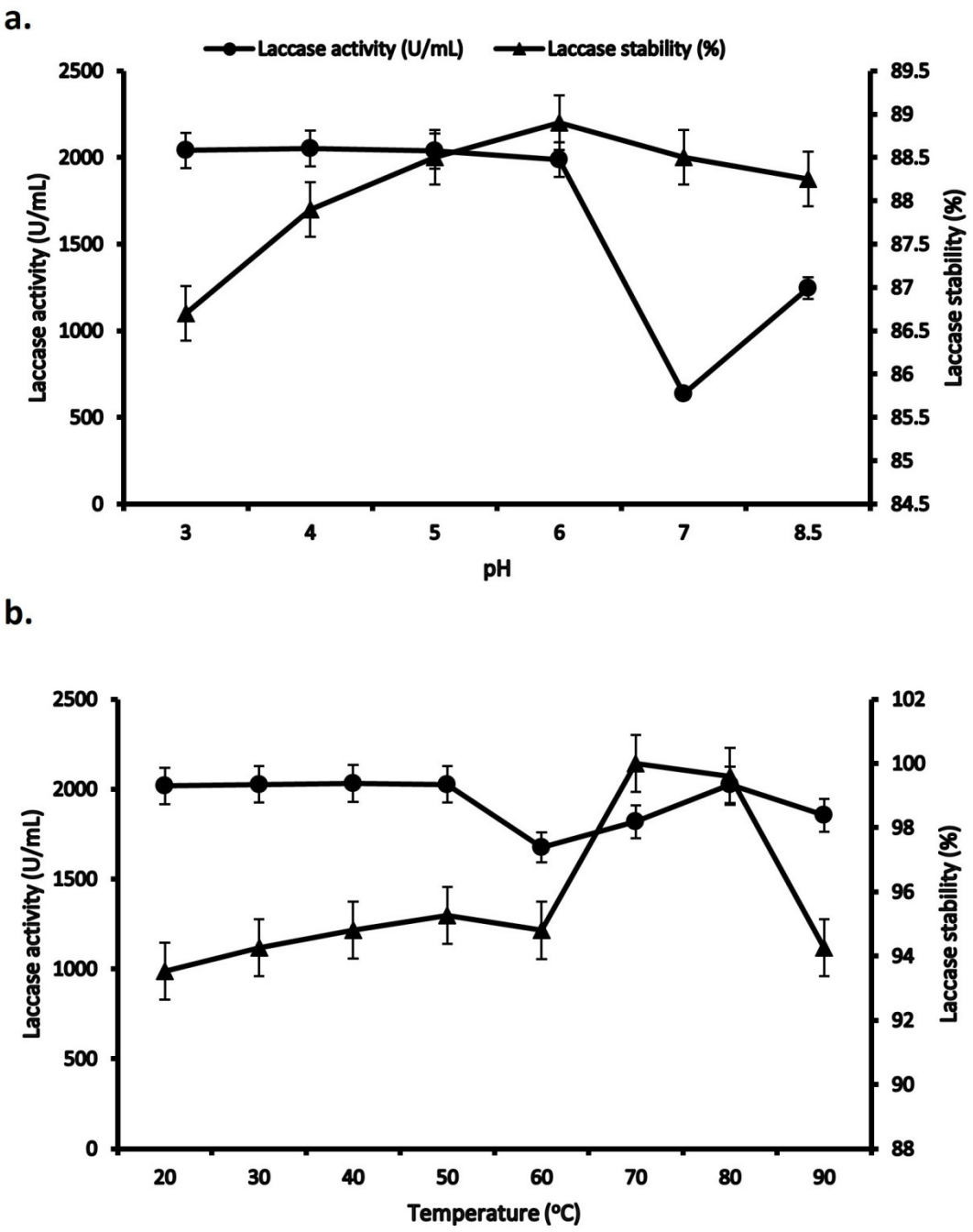

Figure 4 Effect of pH (a) and temperature (b) on partially purified *Trametes* sp. isolate B7 laccase activity and stability.

The ability of fungi to decolourize dyes has been reported in a number of isolates including *Trametes versicolar*. In this study, crude laccase of *Trametes* sp. isolate B7 decolourized synthetic dyes of diverse structures as shown in Fig. 5. Phenol red attained 28% and 36% degradation using 1000 U/mL and 2000 U/mL crude laccase respectively after 48 hours of

incubation. The ability of the crude enzyme to degrade Phenol red without the use of mediators is an important characteristic since Phenol red has a high oxidation potential [51]. This suggests that the crude enzyme possessed the capacity to oxidize a wide range of substrates. Nevertheless, with prolonged incubation of 72 hours to 120 hours the extent of enzymatic degradation dropped. This is because enzymatic degradation of dyes is a multi-step process that involves a decrease in absorbance of the visible peak at the beginning. However, after 72 hours there is a general increase in absorbance due to polymerization of dye fragments resulting in darker solutions [52].

The extent of decolourization of RBBR, Congo red and Malachite green were 100%, 75% and 62% using 1000 U/mL crude laccase and 77.4%, 64% and 28% using 2000 U/mL crude laccase respectively. The degree of decolourization was not the same in all the dyes, probably due to the enzyme concentrations, their substrate specificity as well as the complex structure of many of the synthetic dyes [7]. In a study, 100% of RBBR was decolourized in 6 hours and Congo red in 13 days using 2000 U/mL crude laccase of *T. versicolor* [34]. Interestingly, crude laccase of *Trametes* sp. isolate B7 decolourized 100% of RBBR in 24 hours and 75% of Congo red within 72 hours of the study. These differences are attributed to the fact that the redox potential of enzymes varies with the source which could determine the need of mediators for decolourization of specific dyes [34]. Reports indicate that crude laccase from *L. polychrous* decolourized Rhodamine B and Congo red using the mediator ABTS [53]. However, this study showed that crude laccase from *Trametes* sp. isolate B7 decolourized 75% Congo red without any enzyme mediators. More so, the crude laccase was able to decolouzise Congo red unlike crude laccases from *P. radiata* strain BP-11-2, which failed to decolourize the dye [53].

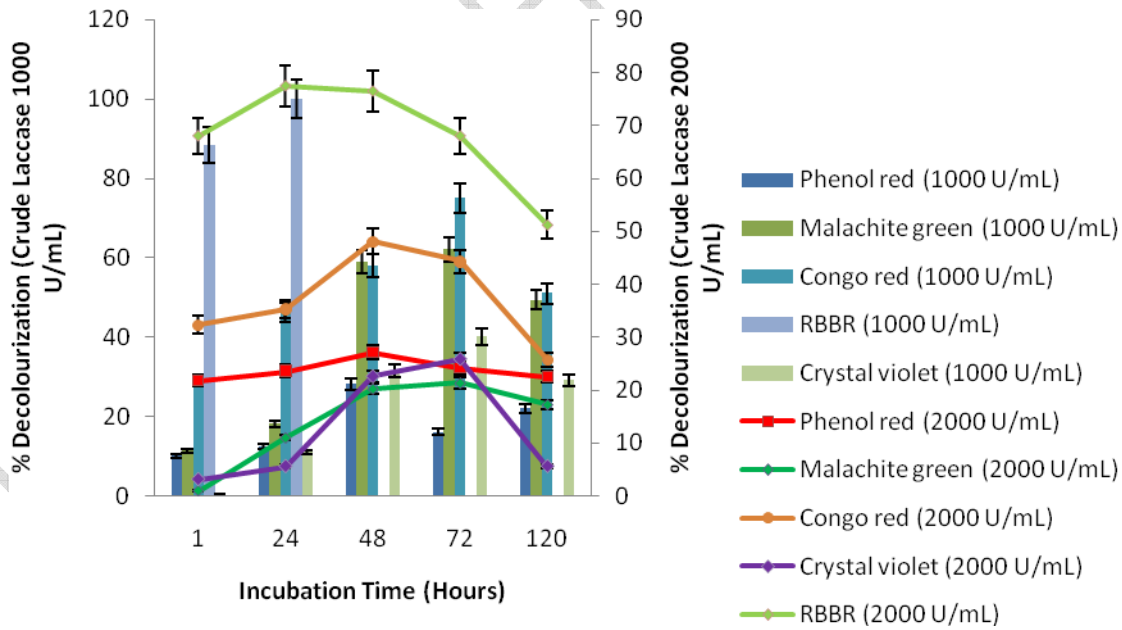

Figure 5 Percentage decolourization of synthetic dyes using 1000 U/mL and 2000 U/mL crude laccase of *Trametes* sp. B7. Bar represent standard error of the mean.

4. CONCLUSION

In the study, plate screening of fungal laccases using ABTS was a rapid, effective and visual method for large-scale sampling of laccase-producing fungi. Therefore, the relatively simple plate screening method proved useful in the discovery of novel laccase of *Trametes* sp. B7. The isolate efficiently produced laccases that were active over a wide pH range, and was 100% thermostable at 70^{o}C for 1 hour. The study also showed that the crude laccase possessed the capacity to oxidize a wide range of synthetic dyes without mediators. Phenol red attained 28% (1000 U/mL) and 36% (2000 U/mL) decolourization using crude laccase. RBBR, Congo red and Malachite green were 100%, 75% and 62% decolourized using 1000 U/mL crude laccase and 77.4% 64% and 28% using 2000 U/mL crude laccase. The ability of the crude enzyme to degrade Phenol red is of biotechnological importance due to its high oxidation potential. This implies that the crude enzyme is capable of oxidizing diverse substrates and of industrial and biotechnological importance.

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