Original Research Article

Screening, Production and Partial Characterization of a Novel Thermostable Laccase of *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 include, low enzyme activity and instability which restrict utilization in many areas of biotechnology. In the study, 59 fungi comprising *Aspergillus niger* (40%), *Aspergillus flavus* (31%), *Aspergillus nidulans* (9.0%), *Trichoderma harzianum* (5.0%), *Trichoderma viride* (5.0%), *Fusarium oxysporum* (5.0%), *Rhizopus stolonifer* (2.0%) and *Trametes sp*. (3.0%) were isolated and screened for laccase production. Plate screening test showed 57.4%, 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 (2356U/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). Decolourization of synthetic dyes were; Phenol red 28% (1000 U/mL), and 36% (2000 U/mL) using crude laccases. 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 a rapid, highly reliable and simple method for large-scale screening of novel laccases with different substrate specificities and improved stabilities. Furthermore, the laccases were highly thermostable, and degraded Phenol red and other synthetic dyes without enzyme mediators. These attribute made the enzyme suitable for application in industry and biotechnology.

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

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 posses 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 [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. The most extensively studied laccases include those from *Agaricus, Trametes* (syn. *Polyporus,* syn. *Coriolus*), *Pleurotus, Podospora, Rhizonia, Neurospora, Aspergillus, Phlebia, Botrytis, Cerrena and Myceliophthora* [3]. Laccases from different organisms exhibit considerable diversity in substrate specificity, molecular weight, temperature and pH optima as well as other properties [7, 8]. 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, phenols, polyphenols, aryl diamines, anilines, hydroxyindols, methoxy substituted phenols, benzenethiols, inorganic/ organic metal compounds and many other xenobiotics [9].

Unlike lignin peroxidase and manganese peroxidase, laccases catalyze the oxidation of organic compounds in the absence of H_2O_2 or Mn^{2+} allowing them to be effectively applied in several industrial processes [10]. Like other enzymes, they often face harsh conditions in industrial processes, such as high temperature, high salt concentration, and extremely acidic or alkaline pH [11]. It has been reported that low yield and enzyme instability are important factors preventing large-scale application of laccases in biotechnology [12]. Thus, screening for laccase-producing fungi is an important way to select suitable organisms capable of producing laccases that could with stand harsh industrial processes. To this end, discovery of novel laccases with different substrate specificities and improved stabilities is necessary for industrial utilizations. Screening of fungal laccases have been carried out either on solid media with coloured indicators that facilitate visual detection, or in liquid cultures which are monitored through measurements of enzyme activities [3]. Generally, solid screening using coloured indicators is simpler because it does not involve sample handling and measurements. Since laccases oxidize various types of substrates, several different compounds have been used as indicators to detect production [3].

Several authors have reported the use of ABTS, guaiacol and *α*-naphthol in plate screening of laccases due to its oxidative potential which results in visual identifications [10]. The oxidation of these presents reddish brown colour in guaiacol, a deep purple colour in *α*-naphthol and a dark green halo around laccase-forming colonies when they react with 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), which are visual confirmations for the secretion of laccases [13]. However, most reports suggest that fungal laccases prefer ABTS as the ideal substrate [10]. Although, the use of ABTS as substrate for screening of laccase provides rapid visualization and confirmation of the enzyme, report indicate the ability of peroxidase enzymes to also oxidize ABTS in the presence of H_2O_2 which might be produced endogenously [14]. As a result, it is necessary to test laccase-producing fungi with ABTS followed by Azure B to confirm that the isolate produces laccase and not peroxidases.

More so, guaiacol can be used to confirm the isolated fungi as laccase-producing but with some doubt in interpreting the results [14]. Therefore, screening with different indicators allows comparison and could reduce the number of indicator compounds required in future for screening procedures.

The pH of a culture medium plays critical role in growth and production of laccase by an organism. One author reported maximum production of fungal laccases when the initial pH of the growth medium was set at pH 5.0 [3]. Most reports have indicated initial pH levels between pH 4.0 - pH 6.0 as optimum for fungal laccases prior to inoculation, however; these levels were not controlled during most cultivations especially in solid state fermentation [3]. Temperature is one of the most critical parameters which plays a vital role in growth and production of laccases by organisms. The optimum temperature for laccase production differs greatly from one strain to another. It has been found that the optimum temperature for laccase production is 25 ^{o}C in the presence of light but 30 ^{o}C when the cultures are incubated in the dark [15].

Thermo-stability is critical to the use of laccases in industry and environmental biotechnology. It varies considerably depending on the source of organism. Generally, studies show that laccases are stable at 30 - 50 ^{o}C and rapidly lose activity at temperatures above 60 ^{o}C [7]. The majority of fungal laccases operate in the range of 30 - 55 ℃, and their optimum pH range is limited to mildly acidic conditions. Many researchers have made attempts to clone novel laccase genes so as to improve their activity and stability. However, the major setback in the use of *E. coli* as a heterologous host is production of catalytically inactive protein aggregates and inclusion bodies [11]. Identification of laccases that are robust to harsh conditions 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 [16]. Laccases are regarded as “Green Tools” because they 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, degradation of synthetic dyes, printing and dyeing industry, bio-pulping in paper industry, conversion of aromatic compounds [17], and removal of phenols which causes cancer and teratogenicity when present in wastewater [18].

Globally, more than 100,000 dyes are commercially available with production of over 7×105 tonnes of the dyestuffs annually [19]. Extensive utilization of these has been reported in diverse areas of industry including textile and leather dyeing, paper printing and coloured photography, cosmetics, food, pharmaceuticals as well as additives to petroleum products [20]. Consequently, close to 10 - 15% of spent dyes are discharged into the environment as wastewater thereby contaminating surface water, soils and underground water by infiltration [21]. Among the many classes of synthetic dyes, the triphenyl methane group of dyes such as crystal violet and malachite green are the largest, and most commonly used in almost every type of application. Most synthetic dyes are characterized by high stability in presence of light and high temperatures, and are fast to detergents and resistant to attacks of microorganisms. Furthermore, they exist in wide arrays of colours and are easy and cheaper to synthesize when compared to natural dyes [22].

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 [23]. Malachite green which is used in dyeing of jute, leather, silk, cotton, wool and ceramics is also highly poisonous to mammalian cells and has been implicated in reproductive abnormalities in fish. It is of public health concern in terrestrial and aquatic

habitats because it may persist in tissues of edible fish through bioaccumulation [24]. The structural similarity of malachite green to other carcinogenic triphenylmethane dyes also raises suspicion of carcinogenicity. Crystal violet on the other hand has been identified as a thyroid and liver carcinogen in rodents while pararosaniline is a bladder carcinogen in humans [25]. Treatments of these pollutants is achieved through conventional methods. Nevertheless, these methods are expensive, time consuming and often lead to formation of toxic residues [26]. Several studies have reported the decolourization of synthetic dyes by fungal species using laccase as the main enzyme during the process [23]. The use of laccase in biological treatment effectively oxidizes pollutants to less soluble compounds that are easily removed by sedimentation and filtration [27] 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 lack toxicity and 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 novel 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 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 [28].

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 [29]. 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 [30]. 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 ^{o}C ± 2 ^{o}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 [31, 32] while those with a dark brown or puplish halos on guaiacol and α-napthol agar respectively were positive for laccase activity [30] 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 [9]. The LMM was autoclaved at 121 ^oC (15 psi) for 15 minutes while 1% (w/v) aqueous glucose solution was separately autoclaved at 110 ^oC (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 $^oC \pm 2$ ^oC for 3, 6, 9 and 12 days.

2.3 Assay of Ligninolytic Enzymes 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 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 [33]. 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.

Lignin peroxidase assay was carried out by the oxidation of veratryl alcohol (3, 4-dimethoxybenzyl alcohol) to veratraldehyde in the presence of H_2O_2 and increase in absorbance measured at 310 nm using spectrophotometer. The reaction mixture consisted 1 mL of 125 mM sodium tartarate buffer (pH 3.0), 500 µL 10 mM veratryl alcohol, 500 µL of 2 mM H_2O_2 solution, and 500 µL of culture filtrate of test strain. Enzyme activity was defined as one unit of enzyme activity that catalyzed the oxidation of 1 µmol of veratraldehyde per minute per mL [34].

Manganese peroxidase activity was measured by the oxidation of phenol red in the presence of H_2O_2. The reaction mixture comprised of 500 µL of the crude enzyme extract, 50 µL of manganese sulfate (2.0 mM), 200 µL of bovine albumin (0.5% w/v), 50 µL H_2O_2 (2 mM) in sodium succinate buffer (0.2 M, pH 4.5), 100 µL of sodium lactate (0.25 M) and 100 µL of phenol red (0.01% w/v). The reaction was monitored by reading the absorbance at 610 nm using spectrophotometer every 10 seconds for five minutes and terminated by adding 40 µL of a sodium hydroxide solution (2.0 M). After termination, the absorbance was monitored for another minute. One unit enzyme was defined as amount to produce 1 µmol product per minute under the experimental conditions (ε_{610} =4460 L, $m^{-1} \cdot cm^{-1}$) [35].

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 [36]. 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 [37].

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 [9]. 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 ^oC for 20 minutes. One percent (w/v) aqueous glucose solution was separately autoclaved at 110 ^oC (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 $^oC \pm 2$ ^oC for 18 days [38].

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 ^oC (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 ℃. 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 [39]. 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 [39]. 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 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 [40]. The enzyme assay was done using the protocol described earlier in 2.3.

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 ^{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 [38, 41].

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 ^{o}C at optimum pH. The enzyme was incubated for 15 minutes and assayed by standard protocol [40].

2.6.4 Effect of temperature on Laccase thermo-stability

Enzyme stability under different temperature was evaluated by incubating the enzyme at various temperatures (20 - 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 [38, 41].

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 ^{o}C ± 2^{o} 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), Rhemazole Brilliant Blue Royal (RBBR) (587 nm) and Malachite Green (620 nm) [27, 42]. 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.

[27].

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 [43].

3. RESULTS AND DISCUSSION

Screenings for laccase production have been primarily carried out in wood rot fungi species of the family basidiomycetes; however, studies in other fungal families are generally lacking. 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* that is native to plant ecosystem. The isolated fungi comprised of *Aspergillus niger* (40%), *Aspergillus flavus* (31%), *Aspergillus nidulans* (9.0%), *Trichoderma harzianum* (5.0%), *Trichoderma viride* (5.0%), *Fusarium oxysporum* (5.0%), *Rhizopus stolonifer* (2.0%) and *Trametes sp.* (3.0%). 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 [44]. 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 [9].

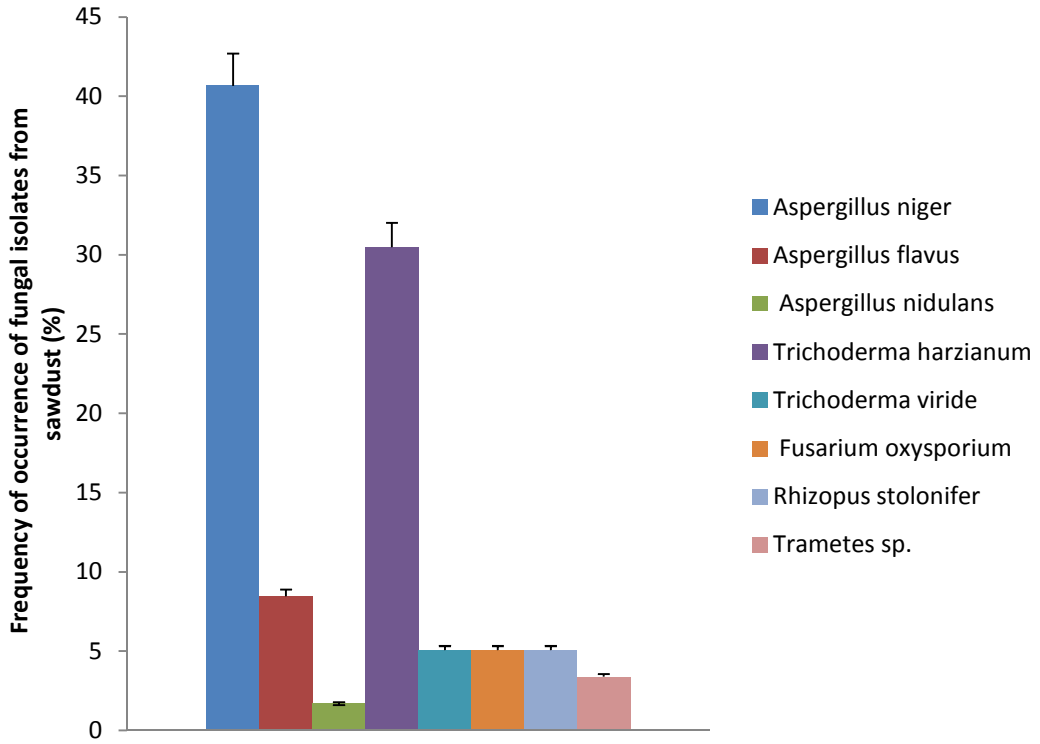


Figure 1 Percentage frequency of occurrence of fungal isolates from sawdust samples of *Terminalia superba.* Bar represent standard error of the mean.

Table 1 shows the qualitative screening of fungal isolates for laccase activity using ABTS, Guaiacol and α-napthol agar. Results showed that 57.4% 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

[10]. Furthermore, laccases from different organisms exhibit considerable diversity in substrate specificity as well as other properties [7, 8]. 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 [14]. 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 [14]. 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 [45].

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 [46]. 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 [44]. 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.

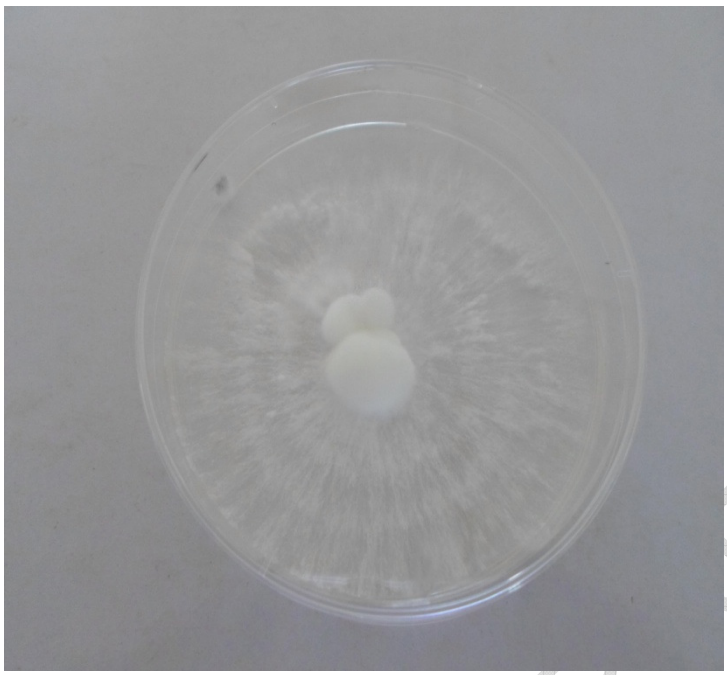
Table 1 Qualitative screening of fungal strains on solid media incorporated with different indicator compounds

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*	-	-	+	-

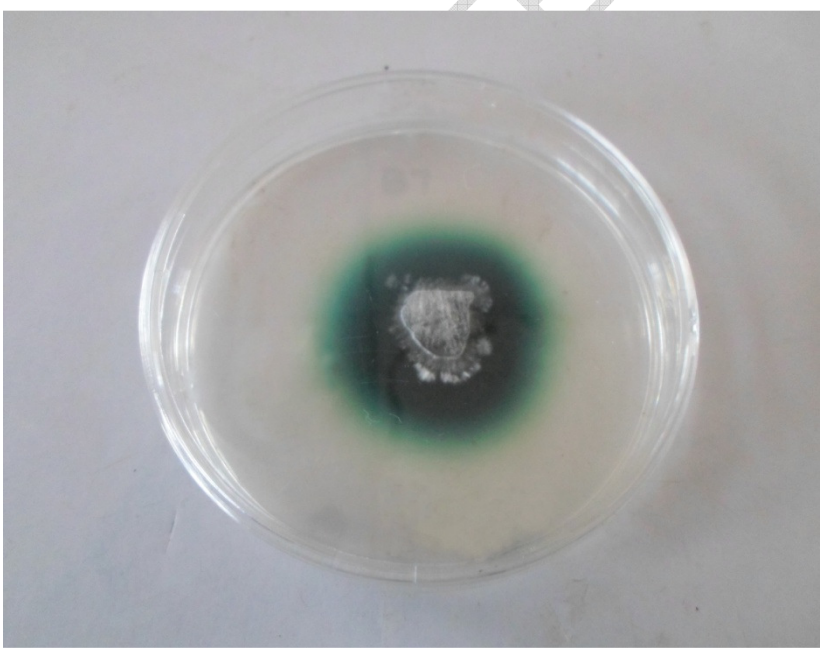
S/No.	Code	Fungi	ABTS	Guaiacol	α-Napthol	Azure B
48	M18	*A. niger*	+	-	+	-
49	M19	*A. niger*	+	-	-	-
50	M20	*A. niger*	+	-	-	-
51	M22	*A. niger*	+	-	-	-
52	M23	*A. niger*	+	-	-	-
53	M24	*A. flavus*	-	-	-	-
54	M25	*T. harzianum*	-	-	-	-
55	M26	*T. harzianum*	-	-	-	-
56	M27	*A. niger*	+	-	-	-
57	M28	*A.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

The use of fungal enzymes in diverse fields has increased the search for efficient and green oxidation technologies to replace the conventional non biological methods. 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 [13, 45, 47]. 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* [17, 47]. Another study using *Fusarium sp., Penicillium sp., Trichoderma* sp. and *Aspergillus* sp. showed oxidation of guaiacol on day four of incubation [44]. 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].

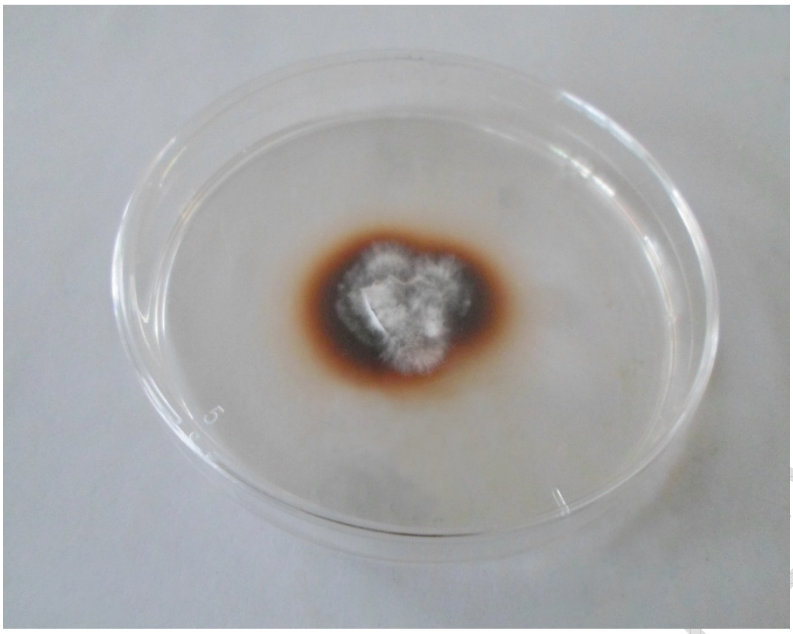
a.



b



c



d

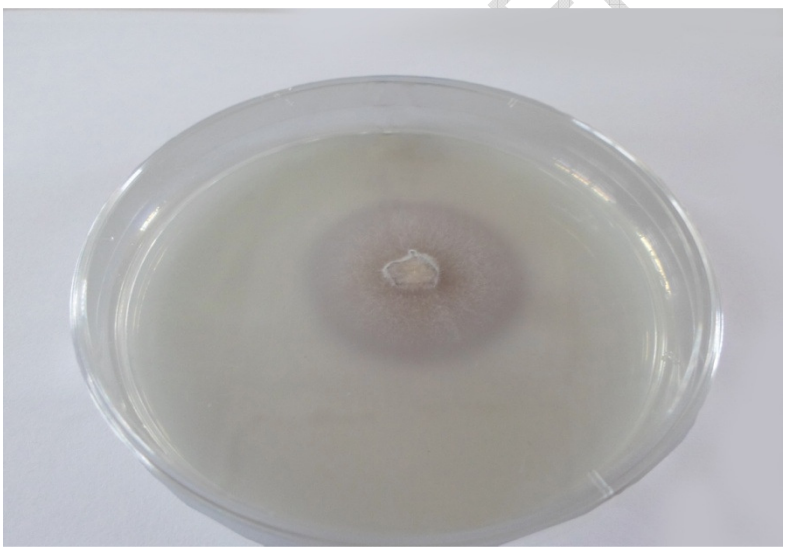


Plate 1a - d shows primary screening of fungal isolate on solid media. (a)*Trametes sp. isolate* B7 on PDA. (b) *Trametes sp. isolate* B7 on ABTS agar showing dark green halo around the colony indicating laccase production. (c) *Trametes sp. isolate* B7 on guaiacol agar showing dark brown halo around 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 [48]. 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 [45]. One author reported production of extracellular laccases in log phase during secondary screening in liquid cultures [48]. Previously, activities of laccases and other ligninolytic enzyme were related to limiting nutrients in the stationary phase of growth in different fungi [49]. It has been established that the actual phase for maximum laccase activity depends on the cultivation medium [50]. 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.

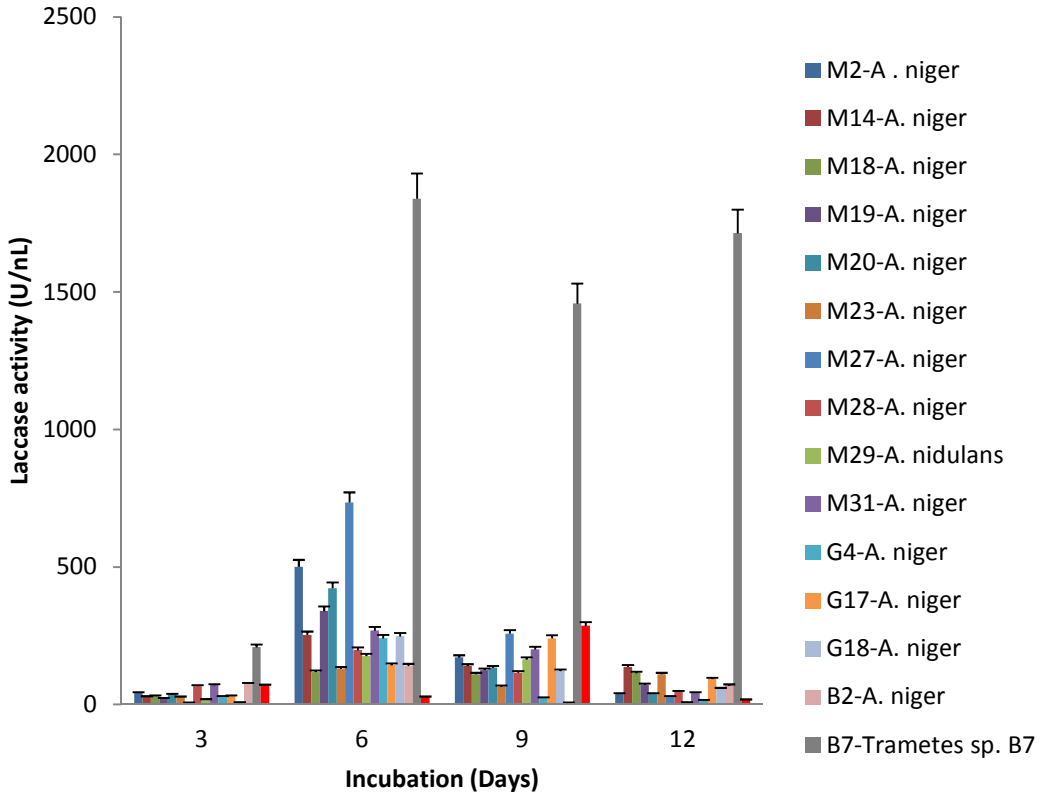


Figure 2. Quantitative screening of fungal strains for laccase production in liquid medium. Bar represent standard error of duplicate determination.

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

Figure 3 shows activities of laccase, lignin peroxidase and manganese peroxidase of *Trametes sp*. in secondary screening. Many reports have documented the production of ligninolytic enzymes by basidiomycetes including *Trametes* sp. Wood Rot Fungi (WRF) are categorised into (i) Lignin peroxidase, Manganese peroxidase and laccase producers, (ii). Manganese peroxidase and laccase producers, (iii). Lignin peroxidase and laccase producers. The most common group is laccase and MnP producers while the rarest group is laccase and LiP producers [51]. Although, lignin peroxidase and manganese peroxidase were not detected during primary screening on solid media, *Trametes sp*. B7 extracellularly secreted these ligninolytic enzymes in liquid cultures. The peaks of all the enzymes were detected on day 6 with 1839 U/mL, 35 U/mL, and 7 U/mL for laccase, lignin peroxidase and manganese peroxidase activities respectively. This is contrary to another study which detected laccase as the only ligninolytic enzyme in a supernatant when the fungus was grown in liquid culture with or without shaking [16]. It is established that some Wood Rot Fungi (WRF) contain all three classes of ligninolytic enzymes, whereas others may contain only one or two of these enzymes [9] which agreed with the study.

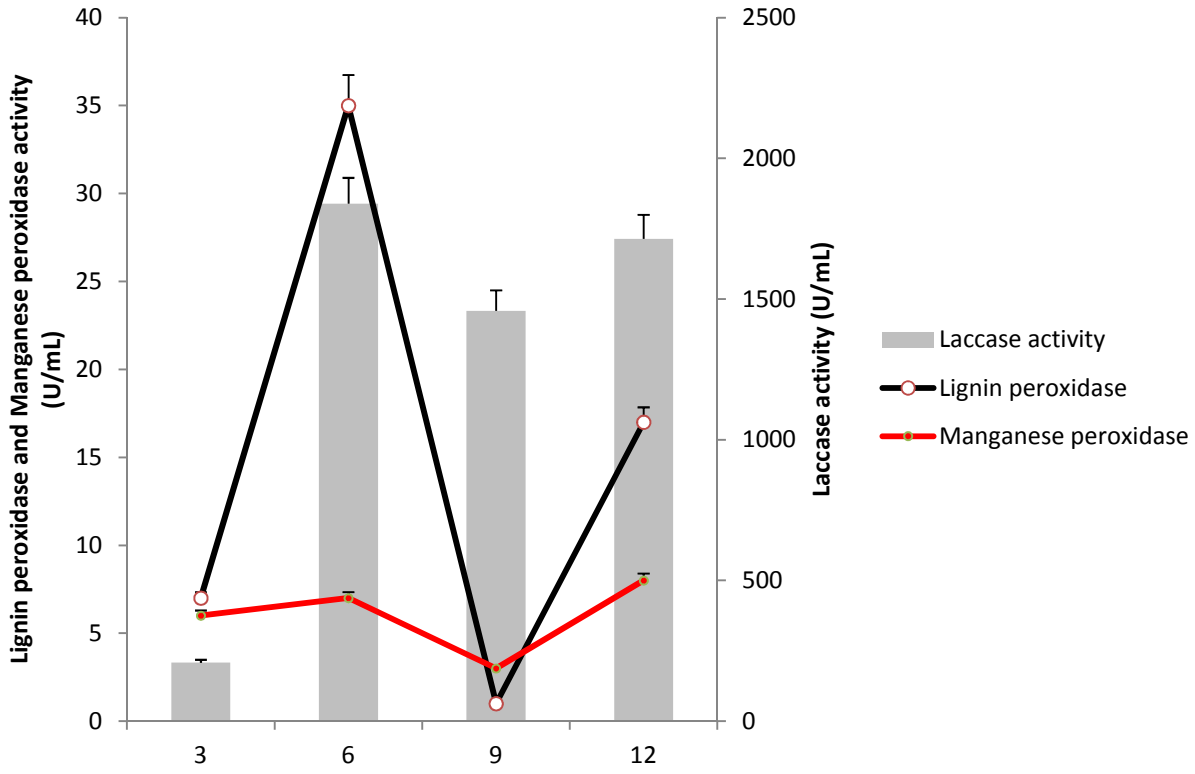


Figure 3 Comparison of laccase, lignin peroxidase and manganese peroxidase activities of *Trametes sp.*B7 during secondary screening. Bar represent standard error of duplicate determination.

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. 4). 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 [52].

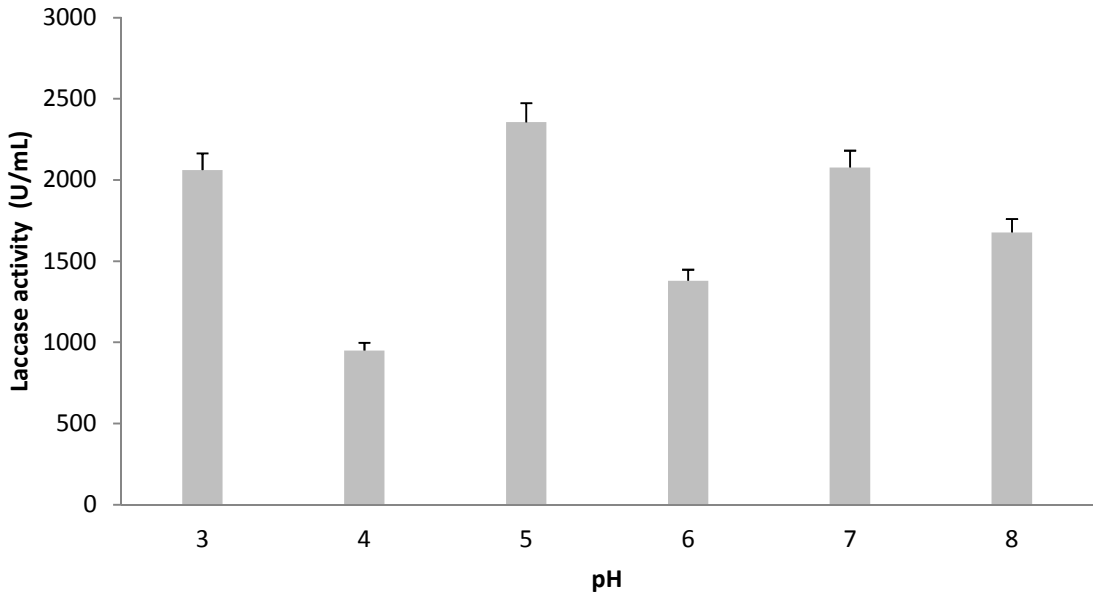


Figure 4 Effect of pH variation on production of laccase. Bar represent standard error of duplicate determination.

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. 5a. 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. [10]. Also, another study reported that most fungal laccases have pH optima in acidic range using ABTS as substrate [11, 53]. 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 [54]. 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 [55]. In addition, characterization of laccase activity from three strains of *Klebsiella pneumoniae* found pH 5.0 as optimum using ABTS [56]. 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 [57]. 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. 5b). Laccase activity was high in the range of 20 - 50 ℃ and 80 ℃. One study reported maximum laccase activity of *T. versicolor* at 40 ℃ [58] 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 [56]. However, laccases of *Cladosporium cladosporioides* were stable from 40 - 70 ℃ but with optimum at

40 °C [54]. The enormous thermostability of *Trametes sp.* B7 laccase makes it more attractive for biotechnological and industrial applications.

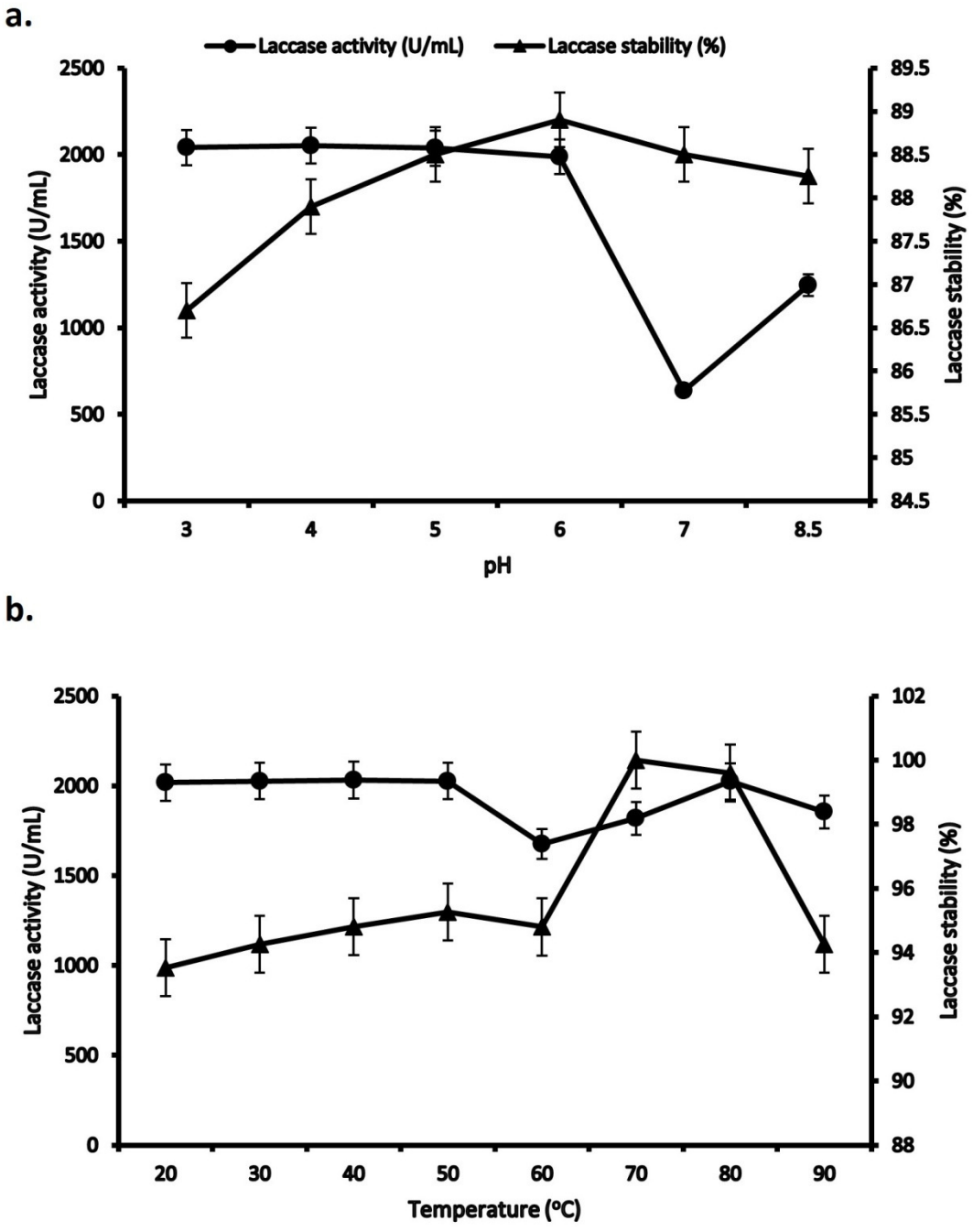


Figure 5 Effect of pH (a) and temperature (b) on partially purified *Trametes sp.* isolate B7 laccase activity and stability.

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. 6. 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 [59]. 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 [60].

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 [9]. 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* [42]. 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 [42]. Reports indicate that crude laccase from *L. polychrous* decolourized Rhodamine B and Congo red using the mediator ABTS [25]. 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 [25].

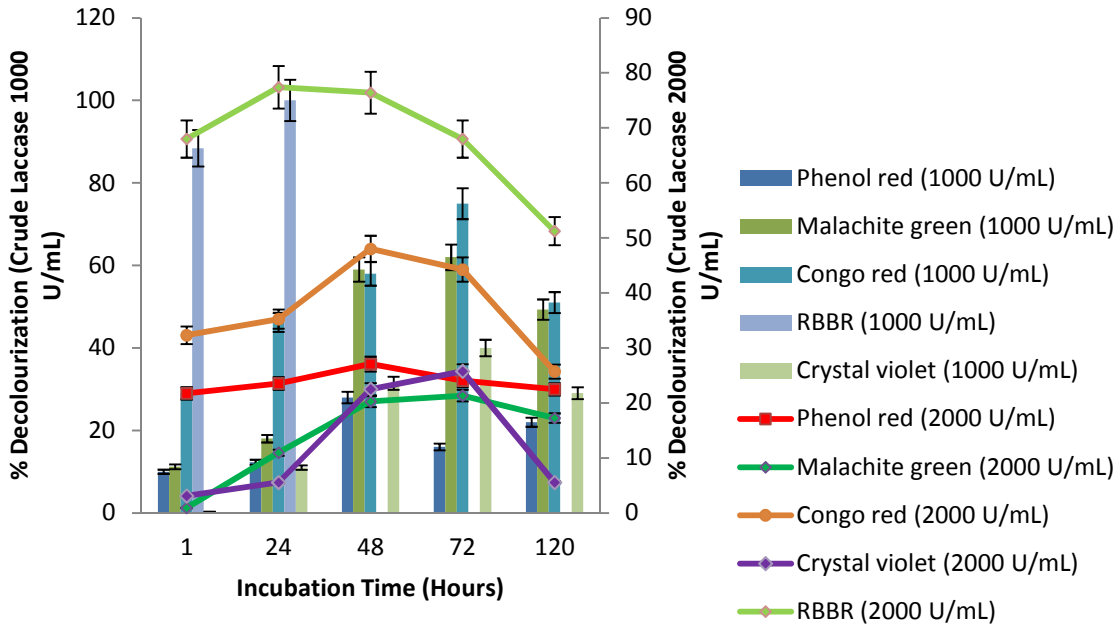


Figure 6 Percentage decolourization of synthetic dyes using 1000 U/mL and 2000 U/mL crude laccase of *Trametes sp.* B7. Bar represent standard error of the mean.

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.

REFERENCES

1. Mate DM, Alcalde M. Laccase: a multipurpose biocatalyst at the forefront of biotechnology. *Microbial Biotechnology*. 2017;10(6):1457-1467.

2. Zhang J, Sun L, Zhang H, Wang S, Zhang X, Geng A. A novel homodimer laccase from *Cerrena unicolor* BBP6: Purification, characterization, and potential in dye decolorization and denim bleaching. *PLOS ONE*. 2018;13(8): e0202440.

3. Viswanath B, Rajesh B, Janardhan A, Kumar AP, Narasimha G. Fungal laccases and their applications in bioremediation. *Enzyme Research*. 2014. DOI.10.1155/2014/163242.

4. Zhao D, Zhang X, Cui D, Zhao M. Characterisation of a novel white laccase from the deuteromycete fungus *Myrothecium verrucaria* NF-05 and its decolourisation of dyes. *Public Library of Science*. 2012;7(6):e38817. DOI: 101371/journal pone 0038817.

5. Souza PM, Bittencourt MLA, Caprara CC, Freitas M, Almeida RPC, Silveira D *et al.* A biotechnology perspective of fungal proteases. *Brazilian Journal of Microbiology*. 2015;46(2):337 - 346.

6. Koyani RD, Rajput KS. Fungal Dynamic strategy and significance for remediating chlorinated. *Journal of Environmental Science, Computer Science and Engineering & Technology.* 2015;5(1):10 - 30.

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

8. Rajeswari M, Bhuvaneswari V. Production of extracellular laccase from the newly isolated *Bacillus sp*. PK4. *African Journal of Biotechnology*. 2016;15(34):1813-1826.

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

10. Ding Z, Peng L, Chen Y, Zhang L, Gu Z, Shi V, et al. Production and characterization of thermostable laccase from the mushroom, *Ganoderma lucidum*, using submerged fermentation. *African Journal of Microbiology Research*. 2012;6(6):1147 - 1157.

11. Ma S, Liu N, Jia H, Dai D, Zang J, Cao Z *et al*. Expression, purification, and characterization of a novel laccase from *Setosphaeria turcica* in *Eschericha coli*. *Journal of Basic Microbiology*. 2018; 58(1): 68 - 75.

12. Asgher M, Wahab A, Bilal M, Iqbal HMN. Lignocellulose degradation and production of lignin modifying enzymes by *Schizophyllum commune* IBL-06 in solid state fermentation. *Biocatalysis and Agricultural Biotechnology*. 2016;6:195 - 201.

13. Shrestha P, Joshi B, Joshi J, Malla R, Sreerama L. Isolation and Physicochemical Characterization of Laccase from *Ganoderma lucidum*-CDBT1 Isolated from Its Native Habitat in Nepal. *BioMed Research International*. 2016;1 - 10.

14. Alfarra HY, Hasali NHM, Omar MN. A lignolytic Fungi with Laccase Activity Isolated from Malaysian local Environment for Phytochemical Transformation Purposes. *International Research Journal of Biological Sciences*. 2013;2(2):51 - 54.

15. Dana M, Khaniki GB, Mokhtarieh AA, Davarpanah SJ. Biotechnological and Industrial Applications of Laccase: A Review. *Journal of Applied Biotechnology Reports*. 2017;4(4): 675 - 679.

16. Sad C, Shanmugam S. Analysis of fungal cultures isolated from anamalai hills for laccase enzyme production effect on dye decolorization, antimicrobial activity. *International Journal of Plant, Animal and Environmental Sciences*. 2012;2(3):143 - 148.

17. Agrawal K, Chaturvedi V, Verma P. Fungal laccase discovered but yet undiscovered. *Bioresources and Bioprocessing*. 2018;5(4):1 - 12. DOI.org/10.1186/s40643-018-0190-z.

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

19. Itodo AU, Oketunde FK. Activated carbon: Spent, Regenerated and Reuse for Synthetic Dyestuff Effluent Decolorization. *International Journal of Environmental Monitoring and Protection.* 2017;4(4), 29 - 37.

20. Gonawalal KH, Mehta MJ. Removal of Color from Different Dye Wastewater by Using Ferric Oxide as an Adsorbent. *Journal of Engineering Research and Applications* 2014;4(5), 102 - 109.

21. Hassaan MA, Nemr AE. Health and Environmental Impacts of Dyes: Mini Review. A*merican Journal of Environmental Science and Engineering*. 2017;1(3):64 - 67. http:// doi: 10.11648/j.ajese.20170103.11.

22. Roushdy MM, Abdel-Shakour EH, El-Agamy EI. Biotechnological approach for lignin peroxidase (LiP) production from agricultural wastes (Rice Husk) by *Cunninghamella elegans*. *Journal of American Science*. 2011;7(5):6 - 13.

23. Songserm P, Sihanonth P, Sangvanich P, Karnchanatat A. Decolorization of textile dyes by *Polyporus pseudobetulinus* and extracellular laccase. *African Journal of Microbiology Research*. 2012;6(4):779 - 792. DOI: 10.5897/AJMR11.988.

24. Fallah AA, Barani A. Determination of malachite green residues in farmed rainbow trout in Iran. *Food Control*. 2014;40.100 - 105. http://dx.doi.org/10.1016/j.foodcont.2013.11.045.

25. Suwannawong P, Khammuang S, Sarnthima R. Decolorization of rhodamine B and congo red by partial purified laccase from *Lentinus polychrous* Lev. *Journal of Biochemistry and Technology*. 2010;2(3):182 - 186.

26. Villegas LGC, Mashhadi N, Chen M, Mukherjee D, Taylor KE, Biswas N. A Short Review of Techniques for Phenol Removal from Wastewater. *Current Pollution Reports*. 2016;2(3): 157 - 167.

27. Moturi B, Singara-Charya MA. Decolourization of crystal violet and malachite green by fungi. Science World Journal. 2009;4(4):1597 - 6343.

28. Domsch KH, Gam W, Anderson TH. Compendium of soil fungi, 2nd Ed., IHWVerlag, Eching, Germany; 2007.

29. Patrick F, Mtui G, Mshandete AM, Kivaisi A. Optimization of laccase and manganese peroxidase production in submerged culture of *Pleurotus sajorcaju*. *African Journal of Biotechnology*. 2011;10 (50):10166 - 10177.

30. Pointing SB. Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi. *Fungi Diversity*. 1999;2:17 - 33.

31. Dhouib A, Hamza M, Zouari H, Mechichi T, H'midi R, Labat M. *et al*. Autochthonous fungal strains with high ligninolytic activities from Tunisian biotopes. *African Journal of Biotechnology*. 2005;4(5):431 - 436.

32. Tapia-Tussell R, Perez-Brito D, Rojas-Herrera R, Cortes-Velazquez A, Rivera-Munoz G, Solis-Pereira S. New laccase-producing fungi isolates with biotechnological potential in dye decolourization. *African Journal of Biotechnology*. 2011;10(50):10134 - 10142.

33. Masalu RJ. Ligninolytic enzymes of the fungus isolated from soil contaminated with cow dung. *Tanzanian Journal of Science*. 2016;42.85 - 93.

34. Kameshwar AKS, Qin W. Qualitative and Quantitative Methods for Isolation and Characterization of Lignin-Modifying Enzymes Secreted by Microorganisms. *Bioenerg. Research*. 2016. DOI 10.1007/s12155-016-9784-5.

35. Silva MLC, de Souza VB, Santos VS, Kamida HM, de Vasconcellos-Neto JRT, Goes-Neto A *et al*. Production of Manganese Peroxidase by *Trametes villosa* on Unexpensive Substrate and Its Application in the Removal of Lignin from Agricultural Wastes. *Advances in Bioscience and Biotechnology*.2014;5:1067 - 1077. doi./10.4236/abb.2014.514122.

36. Ado BV, Onilude AA, Amande TJ. Production and Optimization of Laccase by *Trametes sp.* Isolate B7 and Its Dye Decolourization Potential. *Journal of Advances in Microbiology*. 2018;13(1):1 - 14. DOI: 10.9734/JAMB/2018/44218.

37. Ado BV, Onilude AA, Amande TJ. Utilization of *Terminalia superba* Sawdust as Substrate for Laccase Production by *Trametes sp*. Isolate B7 under Solid State Fermentation. *Microbiology Research Journal International*. 2018;26(3):1-12.DOI: 10.9734/MRJI/2018/45718.

38. Gomes E, Aguiar AP, Carvalho CC, Bonfa MRB, Silva R, Boscolo M. Ligninase production by basidiomycetes strains on lignocellulosic agricultural residues and their application in the decolourization of synthetics dyes. *Brazilian Journal of Microbiology*. 2009;40:31 - 39.

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

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

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

42. Stoilova I, Krastanov A, Stanchev V. Properties of crude laccase from *Trametes versicolor* produced by solid-state fermentation. *Advances in Bioscience and Technology*. 2010;1.208 - 215.

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

44. Kumar VV, Kirupha SD, Periyaraman P, Sivanesan S. Screening and induction of laccase activity in fungal species and its application in dye decolorization. *African Journal of Microbiology Research*. 2011;5(11):1261 - 1267.

45. Patel RJ, Bhaskaran L. Screening of novel ascomycetes for the production of laccase enzyme using different lignin model compounds. *Int J Pharm Bio Sci*. 2016;7(4):452 - 458.

46. Mhaske VR, Wadikar MS. Isolation and screening of *Sclerotium rolfsii* for laccase production. *International Journal of Biology Research*. 2017;2(4):83 - 84.

47. Dabhi BK, Vyas RV, Shelat HN. Biodegradation of lignin by fungal cultures. *Journal of Pharmacognosy and Phytochemistry*. 2017;6(4):1840 - 1842.

48. Gnanasalomi DVV, Gnanadoss JJ. Molecular characterization and phylogenetic analysis of laccase producing fungal isolates with dye decolourizing potential. *Research in Biotechnology*. 2013;4(5):1 - 8.

49. More SS, Renuka PS, Pruthvi K, Swetha M, Malini S, Veena SM. Isolation, purification, and characterization of fungal laccase from *Pleurotus sp*. *Enzyme Research*. 2011;1 - 7. DOI:10.4061/2011/248735.

50. Almeida PH, De Oliveira ACC, De Souza GPN, Friedrich JC, Linde GA, Colauto NB *et al*. Decolorization of remazol brilliant blue R with laccase from *Lentinus crinitus* grown in agro-industrial by-products. *Annals of the Brazilian Academy of Sciences*. 2018;90(4):3463 - 3473. http://dx.doi.org/10.1590/0001-3765201820170458.

51. Lakshmi KMS, Soumya PS, Shaji A, Nambisan P. *Lenzites elegans* KSG32: a novel white rot fungus for synthetic dye decolourization. *Journal of Bacteriology & Mycology.* 2017;5(3):311 - 317.

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

53. Mtibaa R, de Eugenio L, Ghariani B, Louati I, Belbahri L, Nasri M, *et al*. A halotolerant laccase from Chaetomium strain isolated from desert soil and its ability for dye decolourization. Biotech. 2017;7:329. Available:https://doi 10.1007/s13205-017 - 0973-5.

54. Aslam MS, Aishy A, Samra ZQ, Gull I, Athar MA. Identification, Purification and Characterization of a Novel Extracellular Laccase from *Cladosporium cladosporioides*. *Biotechnology & Biotechnological Equipment.* 2012;26(6):3345 - 3350.

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

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

57. Patrick F, Mtui G, Mshandete AM, Johansson G, Kivaisi A. Purification and characterization of a laccase from the basidiomycete *Funalia trogii* (Berk.) isolated in Tanzania. *African Journal of Biochemistry Research*. 2009;3(5):250 - 258.

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

59. Saparrat MCN, Guillen F, Arambarri AM, Martinez AT, Martinez MJ. Induction, isolation, and characterization of two laccases from the white rot basidiomycete *Coriolopsis rigida*. *Applied and Environmental Microbiology*. 2002;68(4):1534 - 1540.

60. Zille A, Gornacka B, Rehorek A, Cavaco-Paulo A. Degradation of azo dyes by *Trametes villosa* laccase over long periods of oxidative conditions. *Applied and Environmental Microbiology*. 2005;71(11):6711 - 6718.