Journal of Advances in Microbiology

13(1): 1-14, 2018; Article no.JAMB.44218
ISSN: 2456-7116

Production and Optimization of Laccase by *Trametes* sp. Isolate B7 and Its' Dye Decolourization Potential

Benjamin Vandelun Ado[1*], Abiodun Anthony Onilude[2] and Tivkaa Amande[3]

[1]Department of Microbiology, College of Science, Federal University of Agriculture, Makurdi, Benue State, Nigeria.
[2]Department of Microbiology, Faculty of Science, University of Ibadan, Ibadan, Oyo State, Nigeria.
[3]Department of Microbiology, Faculty of Science, University of Uyo, Uyo, Akwa Ibom State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author BVA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author AAO managed the analyses of the study. Author TA managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2018/44218
Editor(s):
(1) Dr. Pradeep S., Jawaharlal Nehru Tropical Botanic Garden & Research Institute, Kerala, India.
Reviewers:
(1) Lisa G. A. Ong, Universiti Tunku Abdul Rahman, Malaysia.
(2) Sukhada Akole, Modern College of Arts, University of Pune, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/27227

Original Research Article

Received 30 August 2018
Accepted 23 October 2018
Published 15 November 2018

ABSTRACT

Laccases produced by *Trametes sp*. are extracellular multi-copper oxidases, capable of oxidising large number of xenobiotics including dyes. Challenges often encountered in large-scale utilisation include the high cost of production, low yield and unstable enzymes. Lignocellulosics which abound locally are suitable low-cost alternative substrates for laccase production in Solid State Fermentation. *Trametes sp. isolate B7* (GenBank accession number MK024175) was isolated and identified using molecular techniques. The study optimised laccase production using saw-dust of *Terminalia superba* as well as the application of same for dye decolourisation. Optimal pH and temperature were 5.0 (2356 U/mL) and 25°C (2395 U/mL) respectively. Cu^{2+}, Ca^{2+}, Mn^{2+} and ammonium chloride induced high laccase production at 1-2 mM (2379 U/mL), 3-4 mM (2385 U/mL), 60 mM (2026 U/mL) and 0.3 g/L (2024 U/mL) respectively. Optimum incubation time, carbon

**Corresponding author: E-mail: benjaminado@yahoo.com;*

source and inducer were day 18 (2395 U/mL), glucose (2395 U/mL) and 2,2'-azinobis(3-ethylbenzthiazole-6-sulphonate (ABTS) respectively. The purified laccase had a specific activity of 5830 U/mL and a molecular mass of ~36 kDa using N-PAGE. Dye decolourisation potential of the crude enzyme was RBBR 100% (24 hours), Phenol red 28% (48 hours) while Congo red, Malachite green and Crystal violet were 75%, 62% and 40% respectively after 72 hours. The ability of the crude laccase to oxidise Phenol red and other dyes without the use of mediators is an unusual character and makes it a versatile biotechnological tool for many industrial processes including bioremediation of dyes with resultant eco-friendly benefits.

Keywords: Laccase; Trametes sp; saw-dust; solid state fermentation; optimisation; dye decolourisation.

1. INTRODUCTION

Fungi are well known for their extreme abilities to produce a large variety of extracellular enzymes. Studies have shown that members of Basidiomycetes produce various isoforms of extracellular lignolytic enzymes including laccases which are multi-copper glycoproteins. In culture conditions, laccases are usually the first lignolytic enzymes secreted by fungi into the surrounding media [1]. In many instances, special culture conditions are required for cultivation such as limited carbon and nitrogen levels and use of activators of laccase synthesis to enhance considerable yields [2]. The majority of isolated and characterised laccases are from *Botrytis, Agaricus, Pleurotus, Trametes* (syn. *Polyporus,* syn. *Coriolus*), *Rhizonia, Podospora, Aspergillus, Phlebia, Neurospora, Cerrena and Myceliophthora* [3].

Laccases are easier to manipulate than other lignolytic enzymes such as lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) [4]. Thus, they are widely applied in the degradation of pollutants that cause environmental problems including pulp and mill paper waste, olive mill wastewater, polycyclic aromatic hydrocarbons, chlorinated phenols, polychlorinated biphenyls, dioxins, pesticides, explosives and dyes [5]. However, the challenge has remained that of low enzyme yield and the high cost of production. Therefore, more studies are required on the optimisation of production using cheaper alternative substrates that would lower the cost of production and enhance the industrial application of the enzyme on a commercial scale.

Lignocellulose is the main structural component of both woody and non-woody plants and it constitutes a major renewable source of organic matter [6]. Large amounts of lignocellulosic "waste" such as saw-dust are produced through various activities of man such as forestry and agricultural practices, timber industries, paper-pulp industries and other agro-allied industries which tend to constitute a major problem of environmental pollution. However, their utilization as cheap alternative substrates and optimisation of growth conditions could considerably improve the yield and lower the cost of laccase production [2].

The production of fungal laccases using submerged fermentation has been extensively studied, although in nature these organisms grow in solid-state conditions [7]. However, reports on the use of solid-state fermentation for the production of fungal enzymes are scanty. Reportedly, solid state fermentation (SSF) is an attractive alternative to produce fungal enzymes. This is because the process requires lower capital investment, operating cost and sterility [8]. The process of solid-state fermentation involves a complete or almost complete absence of free liquid. Water, which is essential for microbial activities, is present in an absorbed form within the substrate. These cultivation conditions are particularly suitable for the growth of fungi which grows best at relatively low water activities. As microorganisms in SSF grow under conditions close to their habitats in nature they tend to produce more enzymes and other metabolites which will not be produced or may be produced only in small amounts using submerge conditions [9].

Owing to the diversity of applications of laccases, it's important to identify new sources with high enzyme production. The development of cheap alternatives and high production of this biocatalyst would be attractive due to potential biotechnological applications such as the degradation of dyes with diverse chemical structures including synthetic dyes currently employed in different industries.

This study was designed to meet the demand for low-cost laccase production by *Trametes sp. isolate B7* through optimisation of different procedures using saw-dust of *Terminalia superba* and the application of the crude enzyme to decolourise various polymeric dyes.

2. MATERIALS AND METHODS

2.1 Substrate Collection and Processing

Wood samples of *Terminalia superba* were collected from Gboko plank market, Benue State, Nigeria. Samples were passed through an electric sliding-table saw machine (Makita Precision 2704) to obtain wood blocks which were oven dried to constant weight at 80°C. The blocks were directly fed into a motorized rotary machine (Sawdust machine LT600) and crushed into saw-dust particles. The saw-dust was then passed through a 2 mm wire mesh of metallic sieve to obtain particles of even sizes and thereafter dispensed into plastic bags and sealed.

2.2 Isolation and Molecular Identification of Fungus

2.2.1 Isolation of fungus

The fungus, *Trametes sp. isolate B7* was isolated from decaying wood in Benue Polytechnic Campus, Ugbokolo, Benue State, Nigeria. Pieces of sample were aseptically transferred onto fully sterile Potato Dextrose Agar (PDA) plates and incubated at 27°C ± 2°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°C.

2.2.2 Molecular identification of fungus

Five-day old fungal cultures on PDA plates were observed for both cultural and morphological characteristics [10]. The fungal isolate was sent to Laragen, USA for sequencing. The ribosomal DNA (rDNA) of the fungal strain was extracted and Polymerase Chain Reaction (PCR) amplification of the Internal Transcribed Spacer (ITS) 1-5.8S-ITS2 region of 18S rDNA was performed using ITS 1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS 4 (5' TCC TCC GCT TAT TGA TAT GC 3') primers [11]. PCR conditions included a cycle of initial denaturation at 94°C for 5 minutes, followed by 35 cycles with each cycle comprising of 30 seconds of denaturation at 94°C and 30 seconds of annealing of primers at 55°C and 1.5 minutes extension at 72°C and a final extension for 7 minutes at 72°C in a Primus 96 advanced gradient Thermocycler. The nucleotide sequence obtained was compared to others in GenBank using BLAST [12]. Sequences alignment was carried out using MUSCLE and Phylogenetic analyses by neighbour joining method was performed with MEGA 6.0 software [13,14,15]. The nucleotide sequence obtained was deposited under the GenBank accession number MK024175.

2.3 Production of Growth Medium

The Lignin Modifying Medium (LMM) used to moisten the saw-dust sample 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 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 [16].

2.4 Laccase Production in Solid State Fermentation (SSF)

Ten millilitres of the medium was added to 100 g of saw-dust 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 [17]. The flask was allowed to cool and was aseptically inoculated with two 5 mm agar plugs of actively growing mycelia from 5-day old fungus cultures on PDA and incubated for 18 days. Each experiment was carried out in duplicates.

2.5 Optimization of Laccase Production

2.5.1 Effect of pH on laccase production

The pH of the moistening medium was adjusted to pH values ranging from 3.0 - 8.0 before sterilization and the general procedure performed as described in 2.4. After inoculation, the flasks were incubated at 27°C ± 2°C for 18 days [18,19].

2.5.2 Effect of temperature on laccase production

The fermenting flasks were moistened with LMM (pH 5.0) and the general procedure repeated.

Incubation was carried out at 15°C, 25°C, 35°C, and 45°C for 18 days [18].

2.5.3 Effect of nitrogenous sources on laccase production

2.5.3.1 Effect of peptone and sodium nitrate on laccase production

The effects of peptone and sodium nitrate on laccase production were carried out by substituting 5 g/L of soy tone with 5 g/L of peptone and sodium nitrate separately in the LMM [19,20] before moistening the fermenting flasks. The general procedure was performed at pH 5.0 and incubation carried out at 27°C ± 2°C for 18 days.

2.5.3.2 Effect of ammonium sulphate and ammonium chloride on laccase production

The effect of nitrogen concentration on laccase production was determined by substituting 2 g/L ammonium tartrate with ammonium sulphate and ammonium chloride separately in concentrations of 0.3, 1.0, 3.0 and 6.0 g/L and the medium adjusted to pH 5.0. The general procedure was performed and incubation carried out at 27°C ± 2°C for 18 days [18,19].

2.5.4 Effect of different carbon sources on laccase production

The effect of different sugars on laccase production was carried out by replacing 10 g/L of glucose in the LMM medium with equimolar concentration of the following sugars: lactose, sucrose, maltose and fructose [20]. The general procedure was performed at pH 5.0 and incubation carried out at 27°C ± 2°C for 18 days.

2.5.5 Effect of metal ions on laccase production

To determine the effect of metal ions on laccase production, 10 mL of 1, 2, 3, 4 and 5 mM Ca^{2+}, Cu^{2+} solutions and 20, 40, 60, and 80 mM of Mn^{2+} solution in their chloride forms were added separately to the fermenting flask 48 hours for stability before addition of the moistening medium [21]. The general procedure was performed at pH 5.0 and incubation carried out at 27°C ± 2°C for 18 days.

2.5.6 Effect of activators on laccase production

The effect of ABTS (5 mM), veratryl alcohol (7 mM), guaiacol (10 mM) and glycerol (10 g/L) was determined by adding 7 mL of each activator separately after moistening the fermenting flasks with the LMM (pH 5.0). The general procedure was performed and incubation carried out at 27°C±2°C for 18 days [22].

2.5.7 Effect of incubation time on laccase production

The general procedure was performed at pH 5.0 as earlier described in 2.4 and incubation carried out at 27°C ± 2°C for 6 - 34 days.

2.6 Extraction of Extracellular Enzyme

Extracellular enzymes were extracted by addition of 100 mL of 0.1M citrate-phosphate buffer (pH 5.0) into the fermenting flask. The mixture was stirred with a glass rod for 30 minutes and filtered with cheese-cloth to remove saw-dust and fungal mycelia. The crude filtrate was then filtered with 90 mm Whatman No. 1 Filter paper to obtain a clear filtrate which was refrigerated at 4°C [23].

2.7 Purification and Assay of Laccase Activity

2.7.1 Purification of laccase

The crude extract was centrifuged at 12500 r/min for 25 min at 4°C and the supernatant 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 and the precipitated protein allowed to sediment by repeating the same process of centrifugation. The pellets were collected and resuspended in 50 mL (50 mM, pH 4.5) sodium malonate buffer. The concentrated sample with maximum laccase activity was dialyzed overnight against sodium malonate buffer (50 mM, pH 4.5) using dialysis tubing with Molecular Weigh Cut Off (MWCO) 12 - 14 kDa (Medical Intl. Ltd, 239 Liver Pool, London). The set up was left standing for the initial 2 hours after which the buffer was replaced with a fresh one and dialysis carried out for 24 hours [24]. Enzyme activity was determined before and after dialysis.

2.7.2 One-dimensional native polyacrylamide gel electrophoresis (1-DE N-PAGE)

One dimensional native gel electrophoresis was performed on a vertical slab gel (Mini-PROTEAN II Electrophoresis Cell) with a 12% gradient separating (acrylamide/bis acrylamide) gel and a

4% (w/v) stacking gel although without sodium dodecyl sulphate (SDS). Aliquots (60 µL) of each sample were applied onto each of the gel wells. PAGE Ruler Prestained Protein Ladder (10 to 170 kDa) was loaded onto the first well and was used as the standard. The 1-DE N-PAGE was performed at a constant voltage of 150 V for 6 hours. At the end of the run, activity staining of the gel for laccase was performed with ABTS as the substrate. The non-denatured gel was allowed to stand in 100 mL of sodium acetate buffer (100 mM, pH 4.5) with 1 mL of 10 mM ABTS for 30 min. Laccase activity spots were identified by the development of green coloured bands [25,26].

2.7.3 Assay of laccase activity

Laccase activity was determined spectrophotometrically by following the oxidation of ABTS at 420 nm. The reaction mixture consisted of 600 µL sodium acetate buffer (0.1 M, pH 5.0 at 27°C), 300 µL ABTS (5 mM), 300 µL 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]. 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.

$$Z = \frac{\Delta A.Vt.1000}{\mathcal{E}.Vs.\Delta t}$$

Where

Z = catalytic activity (1µmol per minute)
ΔA= change in absorbance
Vt=final volume of reaction mixture (mL).
$\mathcal{E}$= extinction co-effeicient of ABTS
Vs = sample volume (mL)
t = time in minutes

2.8 Decolourization of Dyes by Crude Laccase

The decolourising potential of crude laccase was tested using selected dyes at a concentration of 0.01% (w/v) dissolved in sterile distilled water. The reaction mixture consisting of equal volume aqueous solution of dye and laccase (1000 U/mL) (1:1) in citrate phosphate buffer (pH 5.0) was incubated at 27°C ± 2°C in the dark for 1, 24, 48, 72 and 120 hours. Decolourisation of dyes was monitored by the decrease in absorbance at the wavelength of maximum absorption for each dye: Phenol Red (475 nm), Methyl Orange (483 nm), Congo Red (497 nm), Methyl Red (535 nm), Crystal Violet (590 nm), Rhemazole Brilliant Blue Royal (587 nm), Malachite Green (620 nm), Azure B (645 nm), and Methylene Blue (660 nm) [28,29]. Control tests were conducted using a heat-denatured crude enzyme. The experiment was carried out in triplicates and decolourisation activity calculated as:

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

Where

D = Decolourisation.
A_{ini} = Initial absorbance.
A_{fin} = Final absorbance of dye after incubation time.

2.9 Statistical Analysis

Results obtained from the study were subjected to analysis of variance using one way ANOVA and differences between means were separated by Duncan Multiple Range Test [30].

3. RESULTS AND DISCUSSION

Molecular identification showed the final fungal sequence of 664 bp long spanning 18S (partial sequence), ITS1, 5.8S, 1TS2 (Complete sequence) and 28S rRNA (partial sequence) region of the DNA. It shared 99% sequence identity with *Trametes sp*. BAB-4765 isolated from mushroom and phylogenetically related to other *Trametes* spp. Therefore, it was referred to as *Trametes sp. isolate B7* (GenBank accession number MK024175).

Fig. 1 present the phylogenetic relationships of *Trametes sp. isolate B7* and selected members of the genera *Trametes* based on ribosomal DNA Internal transcribed spacer (ITS). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the Bootstrap test (1000 replicates) are shown above the branches with Bootstrap values ≥50%.

In this study, laccase production was better at pH 5 with 2356 U/mL as shown in Fig. 2. Many authorities have reported maximum production of laccase by several fungi species including *T.*

versicolor within the range of pH 3.5 - 7.0 which corroborates our finding [19,20]. It is well established that the optimum pH for enzyme production is dependent on the species and strain in addition to the lignocellulosic substrate [31]. From the study, the temperature had the highest effect on production of laccase with 2395 U/mL and 2389 U/mL at 25°C and 30°C respectively (Fig. 3). This agreed with the findings of another study which reported maximum production of laccase by *Trametes* sp., *Pleurotus* sp. and *Dichomitus squalens* at 25°C - 30°C [6]. This is due to the fact that the optimum temperature for an isolate to produce maximal enzyme yield lies close to the optimum temperature for its growth in nature [32].

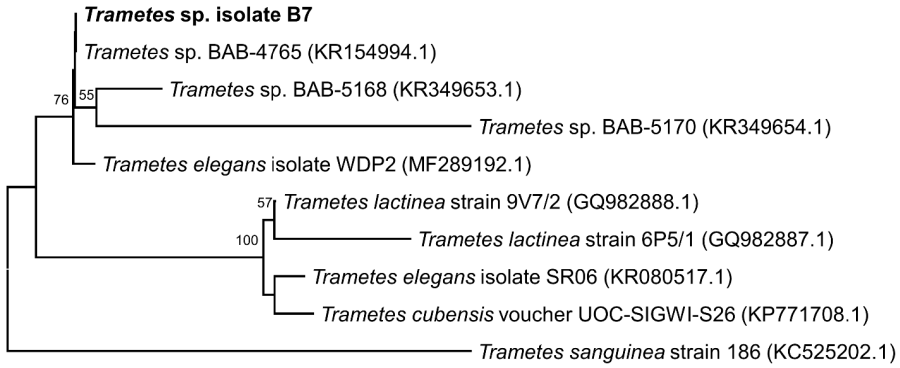

Fig. 1. Phylogenetic relationship of *Trametes sp. isolate B7* with selected members of the genera *Trametes*

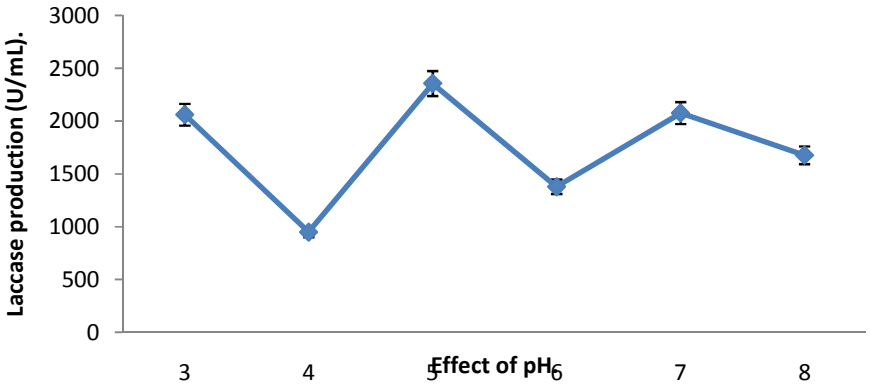

Fig. 2. Effect of pH variation on laccase production. Bar represent standard error of the mean

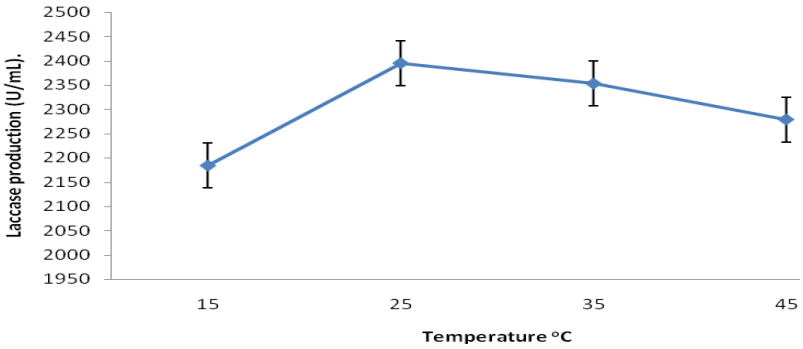

Fig. 3. Effect of temperature variation on laccase production. Bar represent standard error of the mean

The effect of metal ions on laccase production was also investigated. Copper activated high laccase production at 1 - 2 mM with 2379 U/mL beyond which production declined sharply with increase in concentration (Fig. 4). The increase in laccase production may be due to the fact that Cu^{2+} as a co-factor of laccase is required for regulation of gene transcription at a certain threshold beyond which the metal becomes toxic. In addition, at the correct threshold the metal inhibits activities of extracellular proteases which breakdown laccases with a resultant increase in yield [22,33,34]. Another work also reported the stimulatory effect of 1 mM copper chloride on the production of laccase by *T. pubescens* and placed the optimal concentration at 1.5 - 2.0 mM which coincides with our result [35]. The effect of Ca^{2+} on the production of laccase by *Trametes sp. isolate* B7 is shown in Fig. 5. Laccase production increased slightly from 1 mM Ca^{2+} concentration but was highest at 3 - 4 mM with 2385 U/mL beyond which production suddenly declined. This is probably because Ca^{2+} acts as stabilisers which improve the extracellular structures of fungi proteins [21]. It has been established that Mn^{2+} ions act as physiological effectors in fungal cultures. In the study, Mn^{2+} stimulated a steady increase in laccase production from 20 - 40 mM while there was a sharp increase at 60 mM followed by a sudden decline at 80 mM (Fig. 6). The report indicates that maximum biodegradation of wood chips supplemented with Mn^{2+} occurred at 60 - 80 mM using *L. squarrosolus* and *P. atroumbonata* [21]. The slight difference in the peaks of production may be due to differences in sensitivity of the fungal species to the metal ion during growth and enzyme production [36].

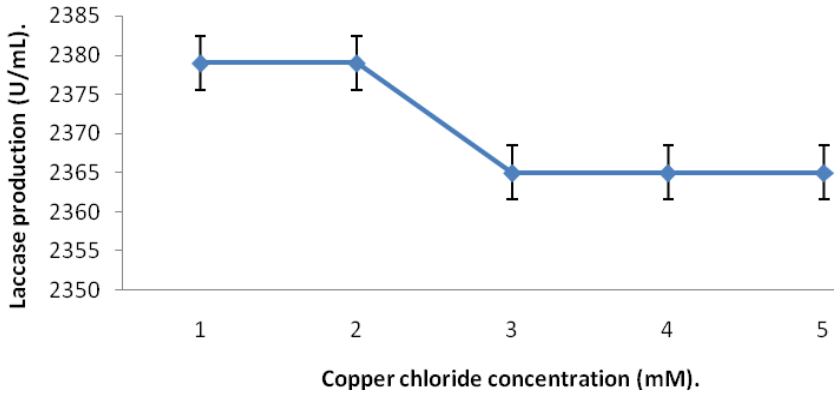

Fig. 4. Effect of different concentrations of copper chloride on laccase production. Bar represent standard error of the mean

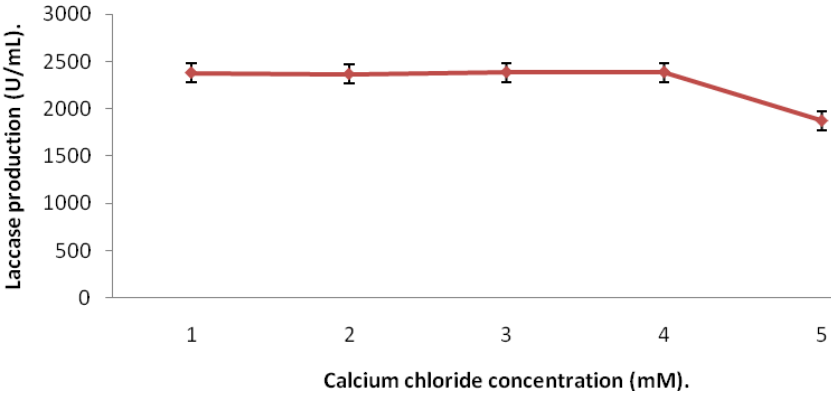

Fig. 5. Effect of different concentrations of calcium chloride on laccase production. Bar represent standard error of the mean

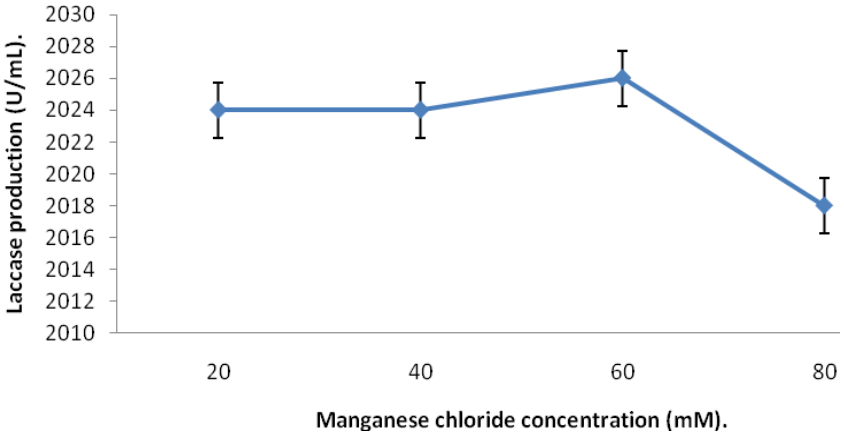

Fig. 6. Effect of different concentrations of manganese chloride on laccase production. Bar represent standard error of the mean

A study on the effect of nitrogenous sources on laccase production was also performed and results obtained as shown in Fig. 7 - Fig. 8. Results showed better activation of laccase production by ammonium chloride at low concentrations of 0.3 - 1.0 g/L with 2024 U/mL compared to ammonium sulphate with 219 U/mL at 1.0 g/L. This is in line with another study which also reported high laccase production with ammonium chloride at lower concentrations contrary to other authorities who stated better stimulation at high concentrations [17]. This variation is probably due to specie specificity. Similarly, LMM supplemented with peptone stimulated the higher production of laccase than sodium nitrate possibly for a similar reason.

The effect of different sugars on laccase production was tested (Fig. 9). Results showed that glucose produced the highest activation of laccase production (2395 U/mL) compared to all the other sugars. Reports indicate that production of laccase by *Coriolus versicolor* at 10 g/L glucose favoured fungal growth and better enzyme production [37] which agreed with the findings of this study. However, laccase production decreased with increased glucose concentration and maximum activity in limited carbon conditions [38]. Another authority reported better production of laccase by *Gonoderma* sp. using starch as the source of carbon [19] probably due to differences in substrate utilisation by different fungi.

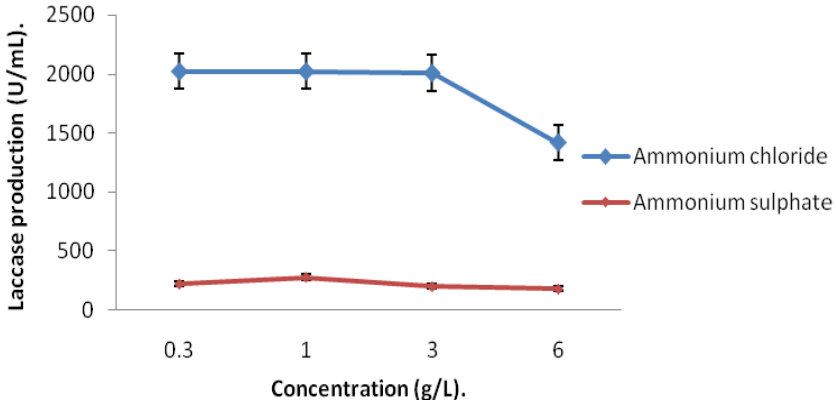

Fig. 7. Effect of different concentrations ammonium chloride and ammonium sulphate on laccase production. Bar represent standard error of the mean

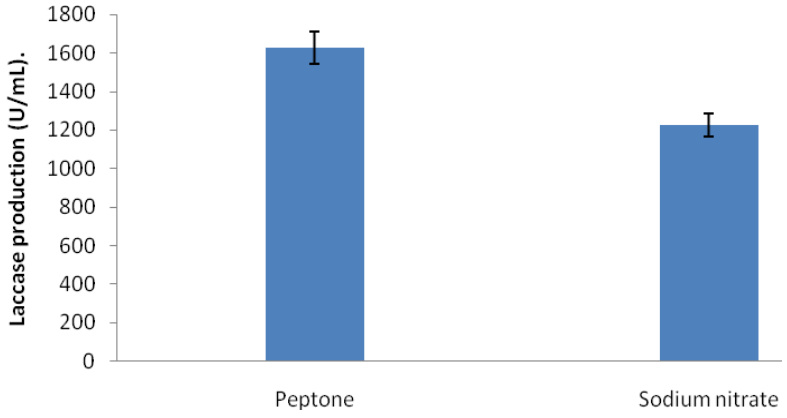

Fig. 8. Effect of peptone and sodium nitrate on laccase production. Bar represent standard error of the mean

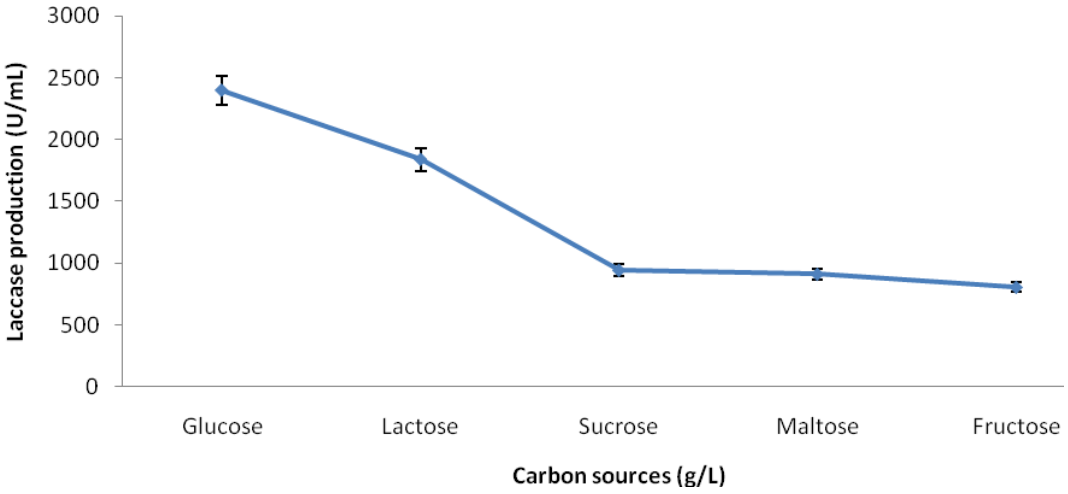

Fig. 9. Effect of different sources of carbon on laccase production. Bar represent standard error of the mean

The ability of different aromatic compounds to induce laccase production was studied and result presented in Fig. 10. Laccase activity was more with ABTS (2110 U/mL) than guaiacol and varytryl alcohol each with 2097 U/mL. Although it is well accepted that aromatic compounds could induce the transcription of the laccase gene and increase laccase production, the effects of aromatic compounds on laccase production by *Trametes sp*. *isolate B7* were less pronounced than those of copper with 2379 U/mL [39].

The study also examined the effect of fermentation period on laccase production as shown in Fig. 11. Enzyme production steadily increased and attained its peak on day 18 after which production declined with increased incubation up to day 34. However, another study reported maximal production of laccase by *P. ostreatus* on day 11 followed by a steady decline with increased incubation. This difference in the optimum day of production was probably because of differences in fungal species [39].

Table 1 present result of purification of crude laccase using ammonium sulphate precipitation and dialysis. The result shows that the specific enzyme activity of laccase increased after purification from 654.44 U/mL, 1487 U/mL, to 5830 U/mL for crude laccase, enzyme pellets

and dialysate respectively with the final purification factor of 9.0.

Plate 1 shows molecular mass of laccase of *Trametes sp. isolate* B7 using N-PAGE and activity staining with ABTS. Most laccases are monomeric glycoproteins showing a molecular mass of between 50 and 80 kDa [40]. However, this study detected a laccase with molecular mass of ~36 kDa which was lower than other reported laccases with 55-65 kDa but higher than laccase of *L. Polychrous* with molecular mass of 32 kDa [41]. However, another work detected two laccases with molecular mass of 38 kDa using SDS-PAGE which was close to our study [42].

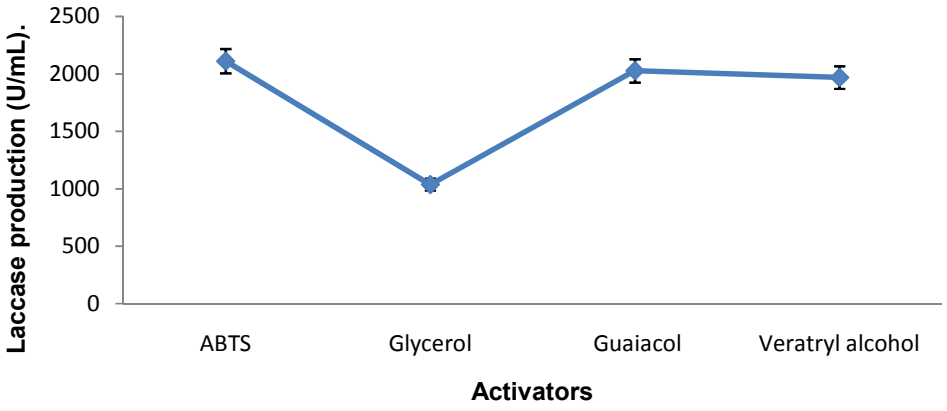

Fig. 10. Effect of different activators on laccase production. Bar represent standard error of the mean

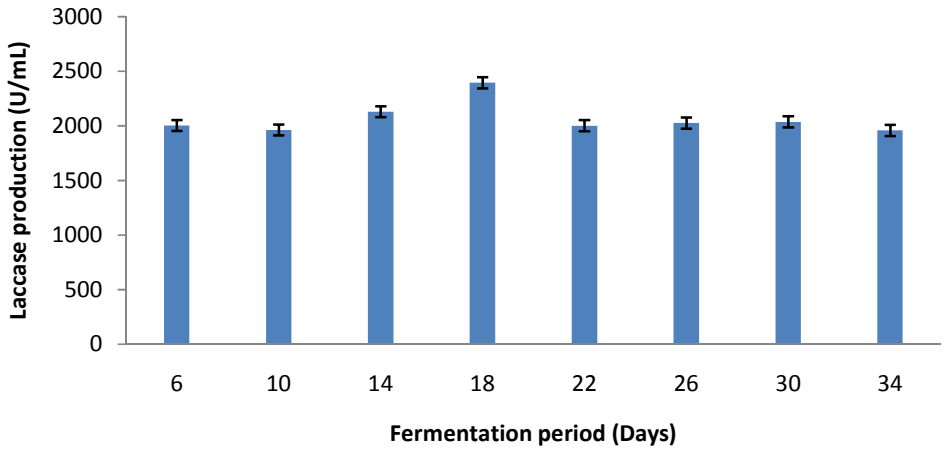

Fig. 11. Effect of fermentation period on laccase production. Bar represent standard error of the mean

Table 1. Purification yield of crude laccase from *Trametes sp. isolate B7* using ammonium sulphate precipitation and dialysis

	Enzyme (Units/mL)	Protein (mg/mL)	Yield (mL)	% Yield	Specific activity	Purification factor
Crude enzyme	2356	3.6	101,900	100	654.44	1
Pellets	2052	1.38	11,080.8	10.87	1487	2.3
Dialysate	2157	0.37	7,224	7.08	5830	9.0

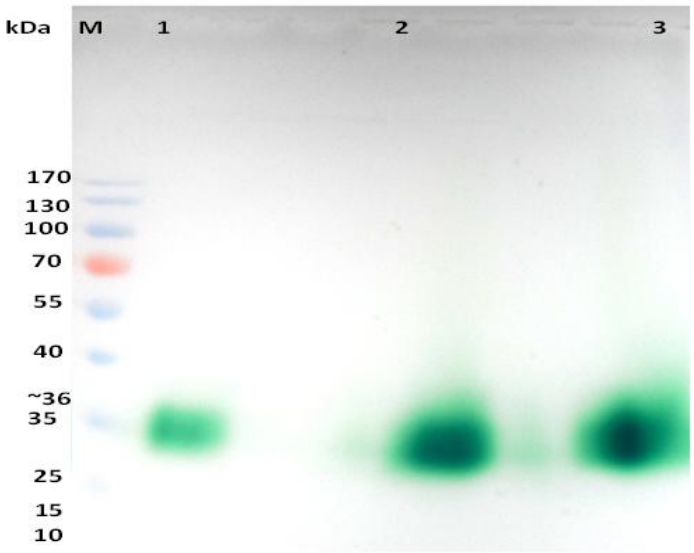

Plate 1. Molecular mass of laccase produced by *Trametes sp. isolate B7* shown on an ABTS-stained N-PAGE

M is the Marker (Prestained protein ladder, 10 – 170 kDa, Invitrogen); 1 is crude laccase; 2 is ammonium sulphate precipitated laccase; 3 is dialysed laccase.

The ability of fungi to decolourise dyes has been reported in a number of isolates including *T. versicolor*. In this study, the ability of crude laccase from *Trametes sp. isolate B7* to decolourise synthetic dyes of diverse structures was assessed as shown in Fig. 12. From the result, 28% of Phenol red was decolourised after 48 hours of incubation using 1000 U/mL of crude laccase. The ability of the enzyme to oxidise Phenol red without the use of mediators is an unusual characteristic of this laccase since Phenol red comparatively has a high oxidation potential [40]. However, decolourisation suddenly declined to 16% at 72 hours. This is because enzymatic degradation of dyes is a stepwise process involving a decrease in absorbance of the visible peak at initial stages of decolourisation followed by a general increase in absorbance after longer periods of treatment up to 72 hours because of polymerization of dye fragments and a resultant darkening of solutions [43].

Decolourisation of RBBR (100%) was achieved after 24 hours while Congo red, Malachite green and Crystal violet successfully achieved 75%, 62% and 40% decolourisation respectively up to 72 hours. Methyl red and Methylene blue attained 70.30% and 25.00% decolourisation at 120 hours respectively. The extent of decolourisation was not consistent in all the dyes probably due to the enzyme system of the fungi, their substrate specificity as well as the complex structure of many of the synthetic dyes [16]. Another study reported 100% decolourisation of RBBR for 6 hours and Congo red for 13 days using 2000 U/mL crude laccase from *T. versicolor* [29]. In this study, crude laccase from *Trametes sp. isolate B7* decolourised 100% of Remazol Brilliant Blue R in 24 hours but also achieved 75% successful decolourisation of Congo red after 72 hours instead of 13 days [29]. These differences account for the fact that the redox potential of laccases varies depending on the source which could also determine the need for a redox mediator during decolouration of specific dyes [29]. Reports indicate that the ability of laccase from *L. polychrous* to decolourise Rhodamine B and Congo red was enhanced in the presence of ABTS and increased with increasing concentrations of ABTS [42]. However, this study showed that crude laccase from *Trametes sp. isolate B7* attained 75% decolourisation of Congo red without the use of enzyme mediators. Moreover, this result contrast with those of laccase from *P. radiata* strain BP-11-2, which did not decolourise Congo red or Methyl orange [42].

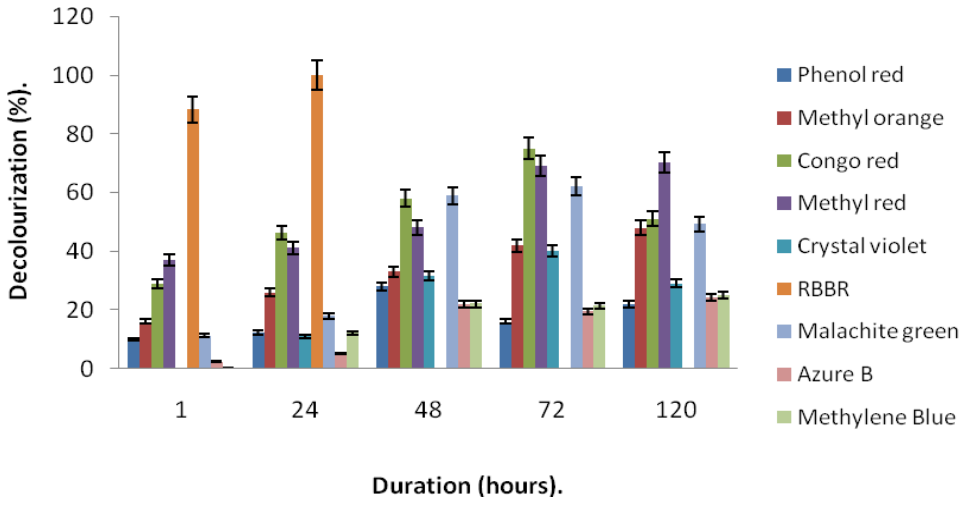

Fig. 12. Decolourisation potential of crude laccase on different polymeric dyes. Bar represent standard error of the mean

4. CONCLUSION

Many recombinant organisms have been successfully utilised in the production of high amounts of various enzymes for large scale commercial applications in industries and bioremediation. However, over- production of laccases in heterogeneous hosts is yet to be achieved. Hence, the necessity to identify and optimise the process of laccase production from another source in nature as carried out in this study. The study established that *Trametes sp. isolate B7* was a high producer of stable laccases using various optimisation procedures including cultural and nutritional parameters. During optimisation, temperature, glucose and incubation time had the highest effect on laccase production in addition to pH. Cu^{2+} a co-factor of laccase and Ca^{2+} equally influenced high production. However, the stimulatory effect of ABTS and other activators on laccase production was generally low. The sawdust of *Terminalia superba* proved to be a useful low cost alternative lignocellulosic substrate for the production of high yield of laccase. The partial purification of crude laccase increased the specific enzyme activity to 5830 U/mL with 7.1% yield and a molecular mass of ~36 kDa. The crude laccase extract demonstrated the potential to catalyse the oxidation of a wide variety of polymeric dyes including phenol red without the use of enzyme mediators; thus, making it a valuable biotechnological tool for many industrial processes including bioremediation of xenobiotics with eco-friendly benefits.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Koyani RD, Rajput KS. Fungal enzymes: Dynamic strategy and significance for remediating chlorinated compounds. Journal of Environmental Science, Computer Science and Engineering & Technology. 2015;5(1):10-30.
2. Viswanath B, Rajesh B, Janardhan A, Kumar AP, Narasimha G. Fungal laccases and their applications in bioremediation. Enzyme Research; 2014. (Accessed 15-8-2016) Available:http://dx.doi.org/10.1155/2014/163242
3. Palonen H. Role of lignin in the enzymatic hydrolysis of lignocelluloses. A Dissertation for the Degree of Doctor of Technology, University of Technology, Espoo, Finland; 2004.
4. Karp SG, Woiciechowski AL, Soccol VT, Soccol CR. Pretreatment strategies for delignification of sugarcane bagasse: A review. Brazilian Archives of Biology and Technology. 2013;56(4):679-689.
5. Machii Y, Hirai H, Nishida T. Lignin peroxidase is involved in the biobleaching of manganese-less oxygen-delignified hardwood kraft pulp by white-rot fungi in the solid-fermentation system. Micro-biology Letters. 2004;233:283-287.

6. Iqbal HMN, Asgher M, Bhatti HN. Optimization of physical and nutritional factors for synthesis of lignin degrading enzymes by a novel strain of *Trametes versicolor*. Bioresources. 2011;6(2):1273-1287.
7. El-Batal AI, El-kenawy, NM, Yassin AS, Amin MA. Laccase production by *Pleurotus ostreatus* and its application in synthesis of gold nano particles. Biotechnol Rep. 2015;5:31-39.
8. Anwar Z, Gulfraz M, Irshad M. Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: A brief review. Journal of Radiation Research and Applied Sciences. 2014;7:163-173.
9. Arotupin DJ, Fabunmi TB, Gabriel-Ajobieve RAO. Enzyme activity of microorganisms associated with fermented husk and testa of *Cola acuminata*. Research Journal of Microbiology. 2015;10(10):466- 475.
10. Domsch KH, Gam W, Anderson TH. Compendium of soil fungi, 2nd Ed., IHW-Verlag, Eching, Germany; 2007.
11. Bakri Y, Masson M, Thonart P. Isolation and identification of two new fungal strains for xylanase production. Applied Biochemistry and Biotechnology. 2010; 162:1626-1634.
12. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Research. 1997;25:3389-3402.
13. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. 1987;4:406-425.
14. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 1985;39:783-791.
15. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution. 2013;30: 2725-2729.
16. 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.
17. 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.
18. Urairuj C, Khanongnuch C, Lumyoung S. Ligninolytic enzymes from tropical endophytic *Xylariaceae.* Fungal Diversity. 2003;13:209-219.
19. Sivakumar R, Rajendran R, Balakumar C, Tamilvendan M. Isolation, screening and optimization of production medium for thermostable laccase production from *Ganoderma sp.* International Journal of Engineering Science and Technology. 2010;2(12):7133-7141.
20. Singh N, Abraham J. Isolation of laccase producing fungus from compost soil and partial characterization of laccase. Advances in Applied Science Research. 2013;4(5):91-98.
21. Wuyep PA, Khan AU, Nok AJ. Production and regulation of lignin degrading enzymes from *Lentinus squarrosulus* (mont.) Singer and *Psathyrella atroumbonata* Pegler. African Journal of Biotechnology. 2003;2(11):444-447.
22. 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.
23. Elsayed MA, Hassan MM, Elshafei AM, Haroun BM, Otham AM. Optimization of cultural and nutritional parameters for the production of laccase by *Pleurotus ostreatus* ARC280. British Biotechnology Journal. 2012;2(3):115-132.
24. 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.
25. Laemmli UK. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature. 1970;227: 680-685.
26. Patel H, Gupte S, Gahlout M. Purification and characterization of an extracellular laccase from solis-state culture of *Pleurotus ostreatus* HP-1. Biotech. 2014.4:77-84.
27. Masalu RJ. Ligninolytic enzymes of the fungus isolated from soil contaminated with cow dung. Tanz. J. Sci. 2016;42:85-93.

28. Moturi B, Singara-Charya MA. Decolourization of crystal violet and malachite green by fungi. Science World Journal. 2009;4(4):1597-6343.
29. 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.
30. Ducan DB. Multiple range and multiple F tests. Biometrics. 1955;11(1):1-42.
31. Elisashvili V, Kachlishvili E. Physiological regulation of laccase and manganese peroxidase production by white-rot *basidiomycetes*. Journal of Biotechnology. 2009;144:37-42.
32. Pang K, Darah I, Poppe L, Szakacs G, Ibrahim O. Xylanase production by a local isolate, *Trichoderma spp.* FETL c3-2 via solid state fermentation using agricultural wastes as substrates. Malaysian Journal of Microbiology. 2006;2(1):7-14.
33. Sadhasivam S, Savitha S, Swaminathan K, Lin FH. Production, purification and characterization of mid-redox potential laccase from a newly isolated *Trichoderma harzianum* WL1. Process Biochemistry. 2008;43:736-742.
34. 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.
35. Stajia M, Vukojevia J, Lausevia SD. Influence of the cultivation conditions on ligninolytic enzyme production in *Pleurotus pulmonarius*. Proceedings Nature and Science. 2007;113:303-312.
36. 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.
37. Kachlishvili E, Metreveli E, Elisashvili V. Modulation of *Cerrena unicolor* laccase and manganese peroxidase production. SpringerPlus. 2014;3:463.
38. Rani V, Mohanram S, Tiwari R, Nain L, Arora A. Beta-glucosidase: key enzyme in determining efficiency of cellulase and biomass hydrolysis. Journal of Bio-processing and Biotechniques. 2014; 5(197):1-8.
39. Zhu XD, Gibbons J, Garcia-Rivera J, Casadevall A, Williamson PR. Laccase of *Cryptococcus neoformans* is a cell wall-associated virulence factor. Infection and Immunity. 2001;69:5589-5596.
40. 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.
41. Majolagbe ON, Oloke JK, Deka-Boruah HP, Bordoloi AK, Borah M. Extraction of extracellular laccase from wild, mutants and hybrid strains of two white-rot fungus and its applications in decolourization and ligninolysis. Journal of Science and Technology. 2012;2:301-317.
42. 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.
43. 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.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/27227