Original Research Article

Production of Fungal Laccase under Solid State Bioprocessing of Agroindustrial Waste and Its Application in Decolourization of Synthetic Dyes

ABSTRACT

Fungal laccases are preferred due to high redox potentials and low substrate specificity to xenobiotics including synthetic dyes. For large-scale applications, low enzyme yield and high cost of production has remained the challenge. Agroindustrial waste such as saw-dust of *Terminalia superba* abounds locally and was utilized as low-cost alternative substrate for laccase production in Solid State Bioprocessing (SSB) using *Trametes* sp. Isolate G31. The study optimized laccase production using various parameters. Optimal production of laccase was at pH 5.0 - 7.0 with 2356 U/mL - 2369 U/mL and 25^{o}C (2336 U/mL). Among the sources of nitrogen and carbon tested, laccase production in ammonium sulphate and sucrose supplemented media were higher. The effect of activators on laccases production showed that Cu^{2+} and Ca^{2+}induced high titres of laccase at 4 -5 mM and 2 - 4 mM respectively, while production of laccase by Mn^{2+} was significantly high at 40 mM. The effect of 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), Guaiacol and Varatryl alcohol on laccase production was not significantly different except in Glycerol. Optimum production for laccase was on day 14 with 2356 U/mL followed by steady declined up to day 34. The purified laccase had a specific activity of 5008, purification factor of 3.85, and a molecular mass of ~40 kDa using N-PAGE. The potential of crude laccase to decolourised diverse dyes was tested. Phenol red achieved 40% decolourization for 1hr, while RBBR (93%), Crystal violet (60%), Methylene blue (53%) and Congo red (51%) for 24 hr, 72 hr, 48 hr and 120 hr respectively. Methyl red and Malachite green attained 42% (72hr) and 32% (48 hr) decolourization. The enzyme ability to oxidise Phenol red and other synthetic dyes without mediators made it eco-friendly in treating dye wastewaters.

Keywords: Trametes sp. isolate G31, Solid State Bioprocessing, sawdust, Optimization, Laccase, Synthetic dyes.

1. INTRODUCTION

Lignocellulose is the major structural component of both woody and non-woody plants and represents a major source of renewable organic matter [1]. Lignocellulosic wastes are generated in large quantities by agricultural practices, paper and pulp, timber and agro industries. The biomass include sawdust, palm kernel shell, rice bran, alfalfa straw, olive pits, wheat straw, rice husks etc. About 140 billion tonnes of lignocellulosic biomass is produced annually world-wide with a substantial part considered as 'waste' [2].

In Nigeria, high annual rainfall promotes forest reserves almost throughout the country. About 11million and 5.5 million hectares of forest and wood lands respectively exist in Nigeria [3]. Wood processing generate huge wastes of which sawmills account for over 93% [3]. Saw-dust is tiny pieces of wood that fall as powder when wood is sawn [4]. Large heaps of sawdust which accumulate at dump sites are often incinerated resulting to air pollution [4]. It also adversely impacts on the soil and water during mulching or when incorporated leading to reduced productivity [4].

However, lignocellulosic biomass consists of cellulose, hemicelluloses and lignin which are valuable components for bioconversion to enzymes and other metabolites in Solid State Bio-processing (SSB) [5, 6]. SSB involves complete or

almost complete absence of free liquid. Water, which is essential for microbial activities, is present in an absorbed form within the substrate. This way, fungus which grows best at relatively low water activities produces more enzymes and other metabolites [7]. SSB is characterized by higher titers of metabolites, low energy consumption, low volumes and cost of equipments, lack of effluents, and utilizes agro-industrial wastes as carbon source making it eco-friendly [6]. Enzyme cost and yield are major factors that justify the economy of SSB for industrial applications.

Fungi as efficient producers of extracellular enzymes are widely applied in environment, industry and food biotechnology. Some Wood Rot Fungi (WRF) secret three classes of ligninolytic enzymes, whereas others produce only one or two [8]. *Trametes versicolar, Trametes* sp. isolate B7, *Pleurotus ostreatus*, *Pleurotus eryngii*, *Agaricus blazei*, and *Clitocybe maxima* secret high titres of laccases while *Ganoderma lucidum* efficiently produces laccases, Manganese and lignin peroxidases [9, 10].

Laccases are multi-copper glycoproteins and were first detected in the sap of a Japanese tree *Rhus* vernicifera [11]. *They* are distributed in a few bacteria, some insects, lichen and fungi [12, 13]. Fungal laccases exist either as extracellular or intracellular enzymes with one or more isoenzymes [11]. They are applied in diverse areas of biotechnology which includes: textile dying or stain bleaching, paper-pulp bleaching, decolourization of synthetic dyes, bioremediation, biosensors, chemical synthesis, immunoassays and the detoxification of contaminated soil and water [14, 15]. Their application is characterized by high enzyme catalysis, less toxicity of intermediate products, shorter treatment regime, and lower cost of energy [16].

Laccase production is affected by nutrient levels, culture conditions, development stages and activities of inducers. These factors vary among fungal species and could regulate isoenzymes of the same strain [17]. The choice of agro-industrial wastes as carbon sources for laccase production is crucial in the economic process [16]. The influence of metals on laccase production is widespread in fungi [17]. Copper induces the transcription of laccase encoding genes in almost all fungi, including *Trametes versicolor*, *Trametes pubescens*, *Pleurotus ostreatus*, *Pleurotus sajor-caju*, and *Ceriporiopsis subvermispora* [17, 18]. Other metals like Mn, Fe, and Zn stimulate similar effects, although the same metals can exert opposing effects in different species.

Dyes are common in textile, food, paper printing and photography, cosmetic, pharmaceutical and leather industries [7]. Generally, reactive dyes are highly soluble, fast to fabrics, stable to light, and resistant to microbial degradation [7]. The discharge of dye wastewaters from several industries is a major environmental challenge. Many synthetic dyes are toxic, carcinogenic or prepared from known carcinogens that could cause harm to humans, animals or other forms of life [19]. Dye suspensions affect aquatic ecosystem and are health concern because of bioaccumulation and soil contamination. Furthermore, their intermediate products are more toxic than the dyes themselves [20].

Treatment of dye wastewater includes physical or chemical adsorption, oxidation, microbial biomass and enzymatic treatments. Nevertheless, the use of microbial enzymes for treatment remains the most effective method [21]. Other methods are ineffective due to partial degradation of dyes, conversion of azo-dyes to toxic metabolites, and conversion of toxic chemicals to secondary solid wastes [22]. More-over, synthetic dyes contain aromatic compounds which can be degraded by microbial enzymes. Fungal laccases are often preferred due to high stability, low substrate specificity and ability to oxidize many phenolic compounds [23].

The main objective of this study was to optimize laccase production by one factor at a time approach using sawdust as a low-cost substrate and application of same for treatment of synthetic dyes.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Fungus

The fungus used in this study was isolated from a saw-dust dump site in Gboko plank market, Benue State, North-Central Nigeria. Pieces of sample were transferred onto sterile Potato Dextrose Agar (PDA) plates and incubated at 27 ± 2 ^{o}C for 7 days. Pure cultures were obtained by sub-culturing onto fresh sterile PDA plates, after which they were placed on PDA slants, and refrigerated at 4 ^{o}C. Five-day old cultures on PDA plates were identified on the basis of cultural and morphological analysis [24, 20].

2.1.1 Plate screening of isolate for laccase production.

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 [25]. The medium was supplemented separately with 0.1% (w/v) 2,2'-azinobis (3-

ethylbenzthiazole-6-sulphonate (ABTS) and Guaiacol followed by 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 [26]. The medium was aseptically transferred into sterile petri dishes (60 mm in diameter) and inoculated with 5 mm agar disk of active fungal mycelia from 5-day old culture. Isolates were incubated at 27 ± 2 ^{o}C for 10 days in darkness. Colonies with dark green halo on ABTS agar and with a diameter above 1 cm on the second day of incubation were considered highly laccase-positive [27].

2.2 Collection and Processing of Substrate

Wood samples of *Terminalia superba* Engl. & Diels were collected from Gboko plank market. The samples were passed through an electric sliding-table saw machine to obtain wood blocks which were oven dried to constant weight at 80 °C. The blocks were directly fed into a motorized rotary machine and crushed into saw-dust particles. The saw-dust was then passed through a 2 mm metallic wire sieve to obtain particles of even sizes which were thereafter dispensed and sealed in plastic bags.

2.3 Production of Growth Medium and Culture Conditions

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 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 [8]. Ten milliliters of the medium was added to 100 g of saw-dust with approximately 70% moisture content in 250 mL Erlenmeyer flask. This was sterilized by autoclaving at 121 ^{o}C for 20 minutes. One percent (w/v) aqueous glucose solution was separately autoclaved at 110 ^{o}C (10 psi) for 10 minutes, and 2 mL aseptically added to the fermenting flask. Flasks were allowed to cool, and 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 in Solid State Fermentation (SSF) at 27 ± 2 ^{o}C for 14 days [28].

2.4 Optimization of Laccase Production

The effect of pH on laccase production was determined by adjusting LMM to pH 3.0 - 8.0 before sterilization, and the general procedure performed as described in 2.3. The effect of temperature was determined by moistening the fermenting flasks with 10 mL LMM (pH 5.0) and incubation carried out at 15°C, 25°C, 35°C, and 45°C [29]. The effects of different nitrogenous sources on laccase production were investigated. Two grams per liter ammonium tartrate was replaced with ammonium sulphate and ammonium chloride, separately in concentrations of 0.3, 1.0, 3.0 and 6.0 g/L in the medium (pH 5.0).Five grams per liter of peptone and sodium nitrate, separately, were substituted for 5 g/L of soy tone in the LMM (pH 5.0) [30,31] and the general process repeated.

Ten grams per liter of lactose, sucrose, maltose and fructose were substituted for 10 g/L glucose in the LMM (pH 5.0) to determine the effect of different sugars [31]. The effect of metal ions were determined by adding 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, separately to the substrate in the fermenting flask, 48 hours for stability before addition of the LMM (pH 5.0) [32]. The effects of ABTS (5 mM), veratryl alcohol (7 mM), guaiacol (10 mM) and glycerol (10 g/L) were determined by adding 7 mL of each activator, separately, after moistening the flasks with the LMM (pH 5.0) [33]. The effect of incubation period was investigated by adding 10 mL LMM (pH 5.0) to 100 g of saw-dust and the general procedure repeated. Incubation was carried out at 27 ± 2°C for 6 - 34 days.

2.5 Extraction of Extracellular Laccase

The 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 using cheese-cloth to remove saw-dust and fungal mycelia. The crude enzyme was then filtered with 90 mm Whatman No. 1 Filter paper to obtain a clear filtrate which was refrigerated at 4°C.

2.6 Purification and Assay of Laccase Activity

2.6.1 Purification of laccase

The extract was centrifuged at 17150 RCF/G for 20 minutes (at 4 ^{o}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 maintained overnight at 4°C and the precipitate allowed to sediment by repeating the same process of centrifugation. The pellets were collected and

reconstituted 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, replaced with fresh buffer and dialyzed for 24 hours [34]. Enzyme activity was determined before and after dialysis.

2.6.2 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) as described earlier [18].

2.6.3 Assay of laccase activity

Laccase activity was determined following the oxidation of ABTS at 420 nm using a spectrophotometer. 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 [35]. 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

2.7 Enzymatic Degradation of Synthetic Dyes

The ability of crude laccase to degrade selected dyes was determined at 0.01% (w/v) in sterile distilled water. The reaction mixture comprised of equal volume aqueous solution of dye and laccase (1000 U/mL) (1:1) in citrate phosphate buffer (pH 5.0). Incubation was at 27 ± 2^{o}C for 1, 24, 48, 72 and 120 hours in the dark. Degradation of synthetic dyes was monitored 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) [36, 37]. Control tests were conducted using a heat-denatured crude enzyme. The experiment was carried out in triplicates and rate of decolourisation calculated as:

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

Where,

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

2.8 Statistical Analysis

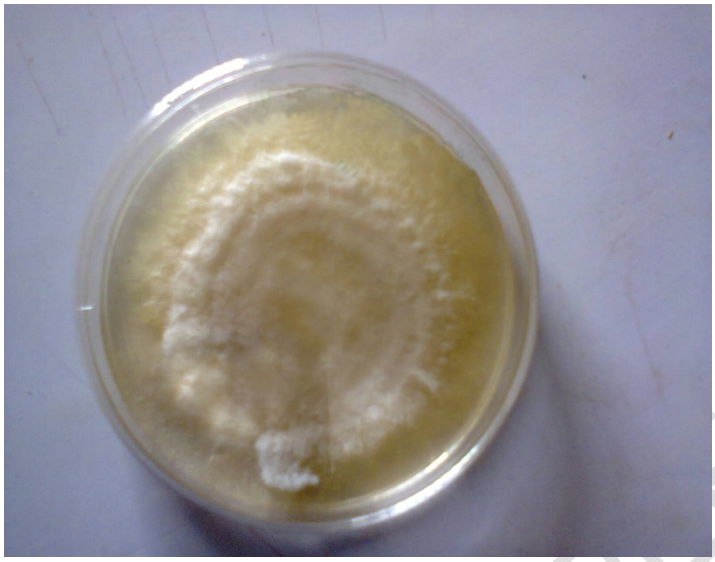
Results obtained from the study were subjected to analysis of variance using one way ANOVA and differences between means were separated by Duncan Multiple Range Test [38].

3. RESULTS AND DISCUSSION

3.1 Isolation and Screening for Laccase Production

The fungus used in this study was isolated from saw-dust dump site and identified. Plate 1a. shows *Trametes* sp. Isolate G31 on Potato Dextrose Agar. The isolate showed laccase activity on ABTS and Guaiacol agar on the second day of incubation by producing a dark green and brown halo around the colony (Plate 1b-c). Several studies have confirmed the activity of laccases in many fungi especially members of the genus *Trametes*. In many studies, different chromogenic indicators such as Guaiacol, Tannic acid, α-Naphthol and ABTS have been used to detect production of laccases in cultures. The use of ABTS and Guaiacol during primary screening for laccase production provides rapid detection and confirmation of the enzyme [10]. It has been established that most laccases of fungal origin show higher sensitivity using ABTS as substrate compared to other chromogenic indicators such as Guaiacol and α-Naphthol [39, 10].

a



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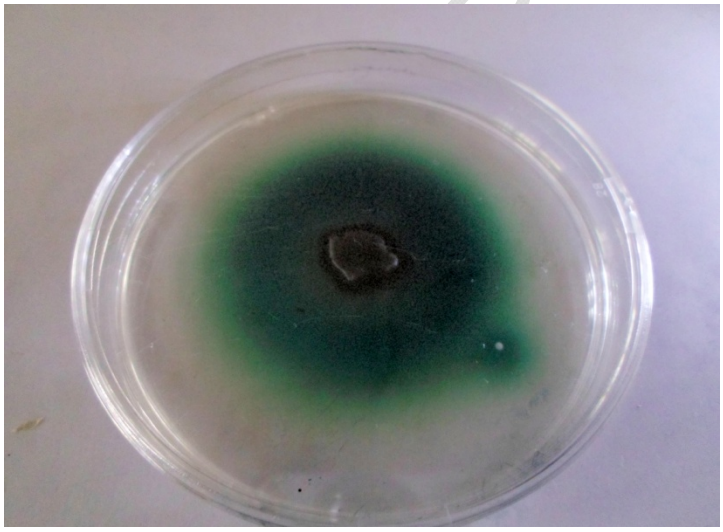

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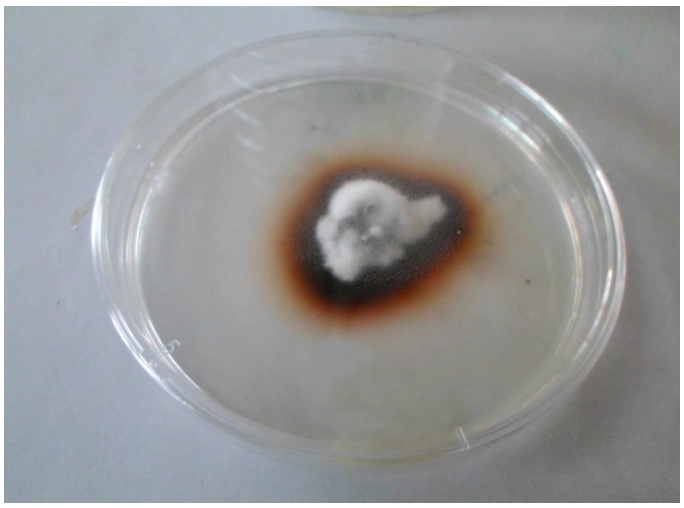

Plate 1(a) *Trametes* sp. isolate G 31 on PDA. (b) *Trametes* sp. isolate G 31 showing a dark green halo on ABTS agar. (c) *Trametes* sp. isolate G 31 showing a dark brown halo on Guaiacol agar.

3.2 Optimization of Laccase Production

3.2.1 Influence of pH and Temperature

The pH of a culture medium plays critical role in growth and production of laccases by organisms. Most reports indicate initial pH levels of 4.0 - 6.0 as optimum for production of fungal laccases prior to inoculation especially in solid state fermentation while others placed the range at pH 3.5 - 7.0 [30, 31]. In this study, pH 5.0 - 7.0 was ideal for optimum production of laccases with 2356 U/mL, 2245 U/mL and 2369 U/mL respectively (Fig. 1). This was consistent with other works which reported optimum laccase production in cultures with slightly acidic pH. For *Pleurotus ostreatus*, *Schizophyllum commune* and *Ganoderma* sp. optimum laccase activity was obtained at pH 5.0, 5.5 and 6.0 respectively [40]. This is because the optimum pH for fungal growth and enzyme production depends on the species and strain as well as the lignocellulosic substrate [41].

The effect of varying temperature on laccase production is presented in Figure 2. Result showed optimum production of laccase at 25^{o}C with 2336 U/mL followed by a decline at 35^{o}C and 45^{o}C with 1956 U/mL and 1811 U/mL respectively. In one study, optimum production of ligninolytic enzyme by *Xylariaceae* occurred at 30 ^{o}C [29]. Similarly, reports on the effect of temperature on laccase production showed the temperature range of 25^{o}C - 30^{o}C as optimum for laccase production by *Trametes* sp. isolate B7, *Pleurotus* sp. and *Dichomitus squalens* [1, 18]. The variation in optimum temperature is probably due to differences in fungal species. This is because the optimum temperature for fungal growth and enzyme production lies close to its temperature in the natural habitat. At these temperatures, fungi membranes are highly permeable while the synthetic machinery function at its peak; consequently, the rate of substrate conversion reaches its maximum [42]. However, higher temperatures tend to denature metabolic activities such as fatty acid synthesis, and membrane fluidity resulting to decrease in production [43].

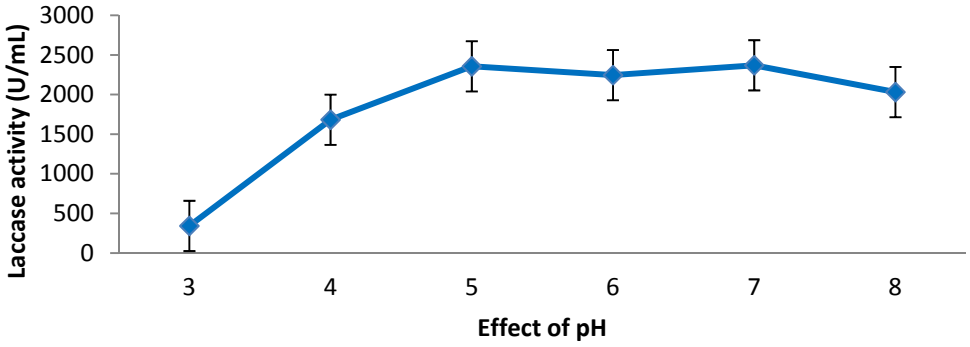

Fig. 1. Influence of varying pH on production of laccase. Bar represent standard error of the mean

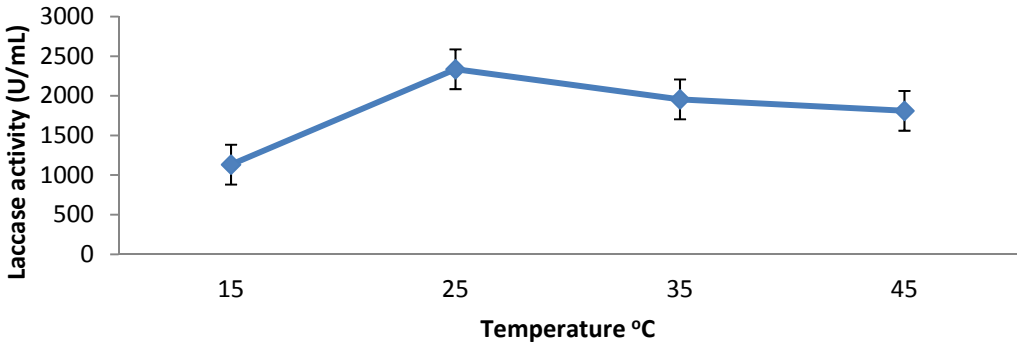

Fig. 2. Influence of varying temperature on production of laccase. Bar represent standard error of the mean

3.2.2 Influence of Nitrogen Sources on Laccase Production

Fig. 3. present the effect of different concentrations of ammonium chloride and ammonium sulphate on laccase production. Ammonium sulphate induced higher production of laccase at 0.3 g/L (2004 U/mL) followed by a sharp declined as concentration increased to 1, 3 and 6 g/L with 1732 U/mL, 706 U/mL and 175 U/mL respectively. However, production of laccase in ammonium chloride supplemented medium increased from 430 U/mL to 1951 U/mL at 0.3 and 1.0 g/L followed by sharp decline of 238 U/mL and 204 U/mL at 3 and 6 g/L respectively. This goes 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 higher concentrations [28] probably due to differences in species. The highest level of enzyme production was obtained with ammonium sulphate which agreed with the study of Stajic *et al*. [44] using *P. ostreatus* HAI 493 and *P. eryngii*. The effect of peptone and sodium nitrate on laccase production showed higher stimulation by sodium nitrate than peptone [Fig. 4]. However, in our earlier study, peptone induced more production of laccase by *Trametes* sp. isolate B7 than sodium nitrate possibly due to species specificity [18].

3.2.3 Influence of Carbon Sources on Laccase Production

Fig. 5. show the effect of different sources of carbon on laccase production. In the study, significant differences in laccase production occurred on all the sugars tested with the highest and lowest productions on sucrose and glucose respectively. Reports indicate that production of laccase by *Coriolus versicolor* and *Trametes* sp. B7 at 10 g/L glucose favoured fungal growth and better enzyme production [18, 45]. However, another study reported better production of laccase by *Gonoderma* sp. using starch as the source of carbon [30]. It has been suggested that sugar such as glucose are easily assimilated and allow constitutive laccase production but tend to repress its induction in several fungi. To avoid this delay time in laccase production, carbon sources that are not very rapidly assimilated are utilized [40]. Undoubtedly, the above variations depend on fungi species and their abilities to use different sources of carbon.

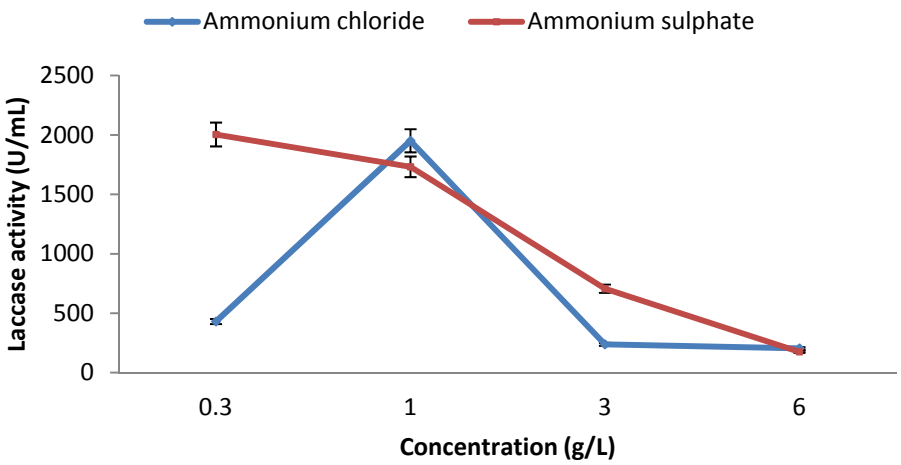

Fig. 3. Influence of different concentrations of ammonium sulphate and ammonium chloride on laccase production. Bar represent standard error of the mean

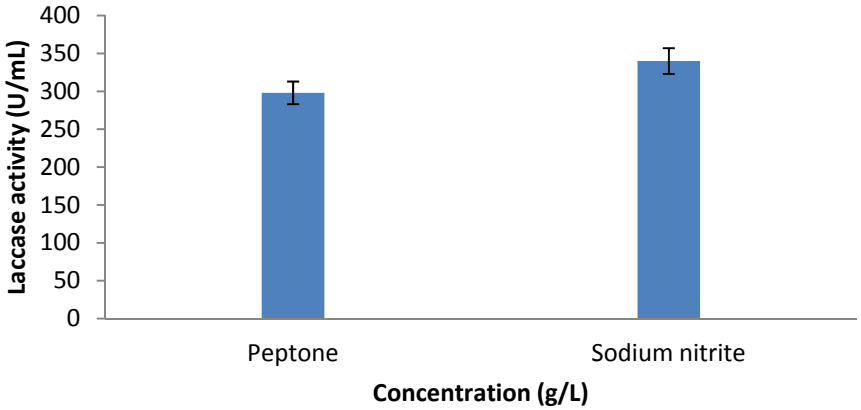

Fig. 4. Influence of peptone and sodium nitrate on production of laccase. Bar represent standard error of the mean

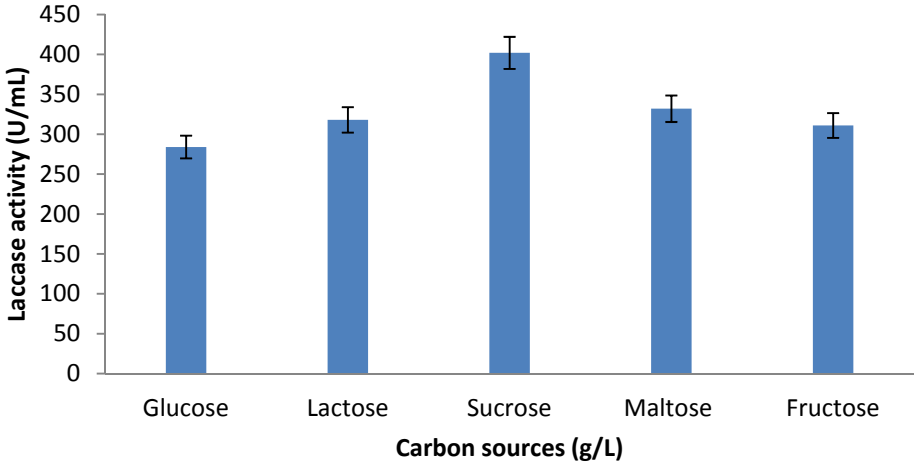

Fig. 5. Influence of different carbon sources on production of laccase. Bar represent standard error of the mean

3.2.4 Influence of Metal ions on Laccase production

The effect of Cu^{2+} ions on laccase production is presented in Figure 6. Laccase production increased with concentration of Cu^{2+} ions and optimum activity of 2127 U/mL and 2131 U/mL at 4 - 5 mM respectively. The regulatory effect of copper on

transcription of genes for laccase production has been reported. In addition, copper ions are known to inhibit extracellular production of proteases which degrade laccases thereby resulting to increase in laccase activity and stability [33, 44]. Studies have shown the stimulatory effect of Cu^{2+} ions on production of laccase by *Trametes pubescens*, *Trametes trogii*, *Pleurotus ostreatus and Trametes* sp. isolate B7 in low concentrations and with optimum activation at 1 - 2 mM [18, 44, 46]. On the contrary, this study showed that production of laccase by *Trametes* sp. G31 increased with concentration and maximum enzyme activity was detected at 4 - 5 mM. In another study, laccase production by *Pleurotus eryngii* increased with Cu^{2+} concentration up to 70 µM after which it decreased [7]. These differences in optimal Cu^{2+}ion concentration could be due to differences in sensitivity of the isolates to the metal ions [7].

Fig. 7. shows the effect of Ca^{2+} on production of laccase. In the study, high activities of laccase were reported at 2 - 4 mM with 2077 U/mL, 2093 U/mL and 2032 U/mL respectively, and a decline at 1 and 5 mM. It has been reported that Ca^{2+} enhances laccase activity because it improves the extracellular structure of fungal proteins and act as stabilizers [32]. However, the effect of Cu^{2+} ion on laccase production was significantly higher than Ca^{2+} ion since copper-mediated regulation of laccase occurs at the transcription level [17]. It has been established that Mn^{2+} ions act as physiological effectors in fungal cultures. In the study, Mn stimulated increase in laccase production from 20 mM (1459 U/mL) to 40 mM (1652 U/mL) while there was a sharp decrease in production at 60 - 80 mM (Fig. 8). However, another study reported maximum biodegradation of wood chips supplemented with Mn^{2+} at 60 - 80 mM using *L. squarrosolus* and *P. atroumbonata*. The obvious difference in the peaks of production may be due to differences in sensitivity of fungal species to the metal ion during growth and production of enzymes on lignocellulosic substrate [47].

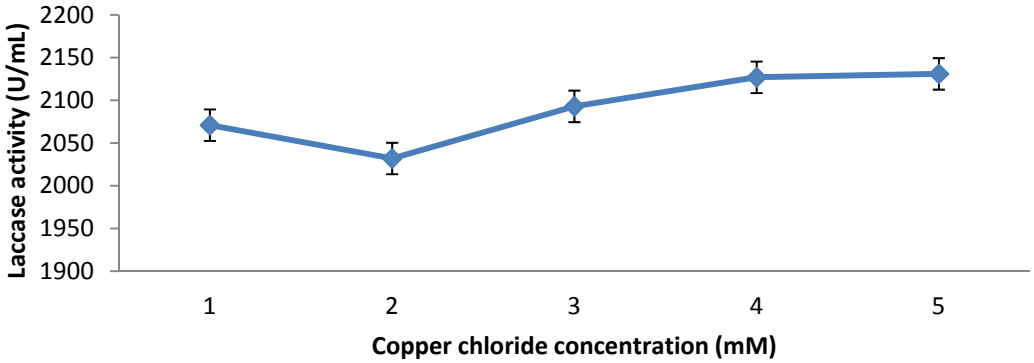

Fig. 6. Influence of different concentrations of copper chloride on production of laccase. Bar represent standard error of the mean

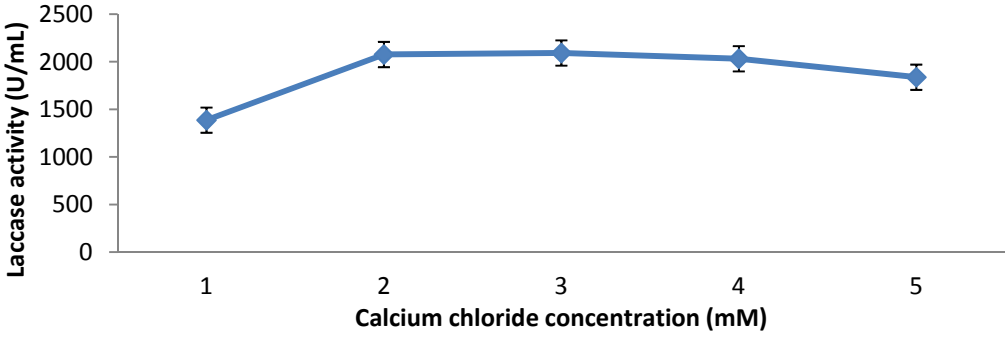

Fig. 7. Influence of different concentrations of calcium chloride on production of laccase. Bar represent standard error of the mean

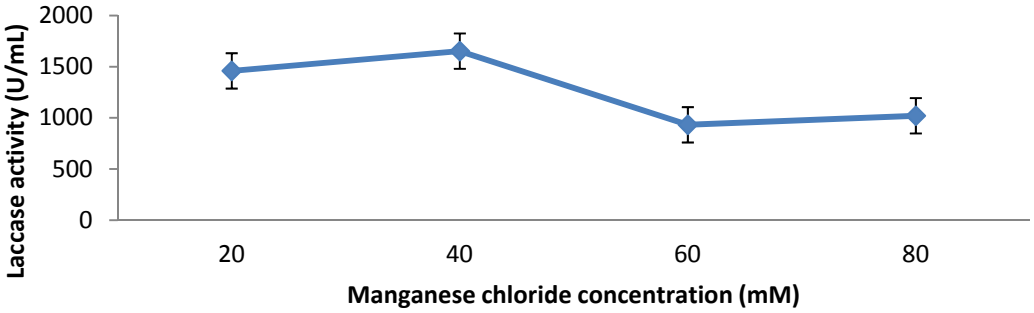

Fig. 8. Influence of different concentration of manganese chloride on production of laccase. Bar represent standard error of the mean

3.2.5 Influence of Activators on Laccase Production

The ability of different compounds to activate laccase production was studied and result presented in Fig.9. Production of laccase has been found to be highly dependent on the culture conditions and composition of the nutrient medium [48]. In the study, laccase activity was slightly higher on ABTS than guaiacol, varytryl alcohol and glycerol. Even though it was expected that afore-mentioned compounds could induce the transcription of the laccase gene and increase laccase production, their influence on production of laccase by *Trametes* sp. G31 was much less when compared to copper as seen in our earlier study [18]. In another study, Ire and Ahuekwe [48] tested four compounds for laccase production namely ABTS, Tween 80, Soya oil and Malachite green and reported that production of laccase by ABTS was the lowest while Soya oil and Tween 80 showed remarkably high productions. This was probably because Tween 80 and Soya oil increased the bioavailability of less soluble substrates for fungal growth [49].

3.2.6 Influence of Incubation Period

The influence of incubation period on production of laccase was examined and result presented as shown in Fig. 10. Production of laccase increased from day 6 - 10 with 1721 U/mL and 1983 U/mL respectively and attained a peak of 2356 U/mL on day 14. Thereafter, production declined gradually with increased incubation up to day 34. In another study, maximum production of laccase by *P. ostreatus* occurred on day 11 while production of laccase by *Trametes* sp. B7 on *Terminalia superba* was highest on day 18 followed by a steady decline with increased incubation. These differences in the optimum day for laccase production are probably because of differences in fungal species as well as their abilities to utilize different substrates [50].

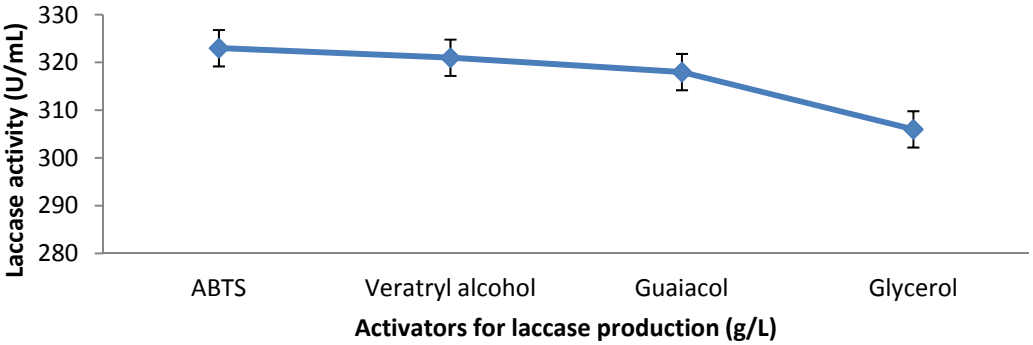

Fig.9. Influence of different activators on production of laccase. Bar represent standard error of the mean

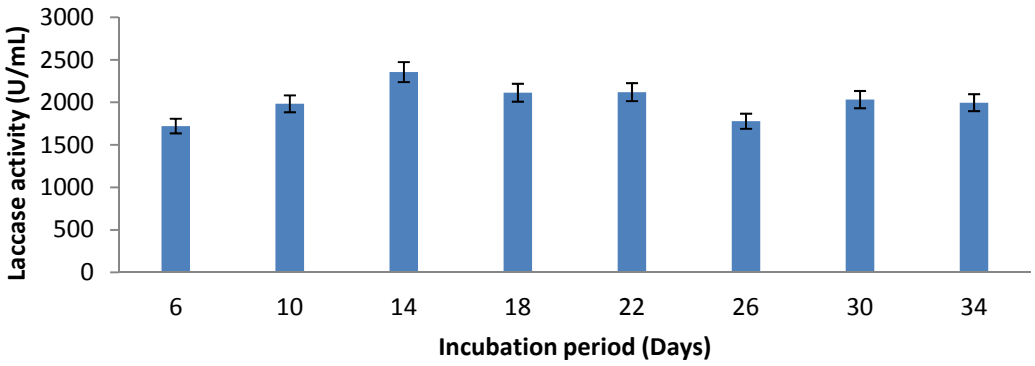

Fig. 10. Influence of incubation period on production of laccase. Bar represent standard error of the mean

3.3 Purification of Laccase

Purification of crude laccase using ammonium sulphate precipitation and dialysis was carried out and result presented in Table 1. The result shows that crude laccase and specific activities were 2077 U/mL and 1298.13 respectively. During ammonium sulphate precipitation laccase activity of the pellets decreased to 1996 U/mL while the specific activity increased to 3118.75 and purification factor to 2.40. Upon dialysis, laccase activity further declined to 1753U/mL, specific activity increased to 5008.57 with the enzyme 3.85 times purified.

Plate 2 presents the molecular mass of laccase of *Trametes* sp. G31 using N-PAGE and stained with ABTS. It has been reported that most laccases are monomeric glycoproteins which possess molecular masses within the range of 50 - 80 kDa [51]. This study detected a laccase with molecular mass of ~40 kDa which was lower than the 55 - 65 kDa reported for other laccases but higher than the 32 kDa laccase of *L. Polychrous* [52]. However, other works detected two laccases with molecular mass of 36 kDa and 38 kDa which were close to our study [l8, 53].

Table 1. Ammonium sulphate purification and dialysis of crude laccase from Trametes sp. G31

	Enzyme (Units/mL)	Protein (mg/mL)	Yield (mL)	%Yield	Specific Activity	Purification Factor
Crude enzyme	2077	1.60	103,85	100	1,298.13	1.0
Pellets	1996	0.64	23,95	23	3,118.75	2.40
Dialysate	1753	0.35	8765	8.44	5008.57	3.85

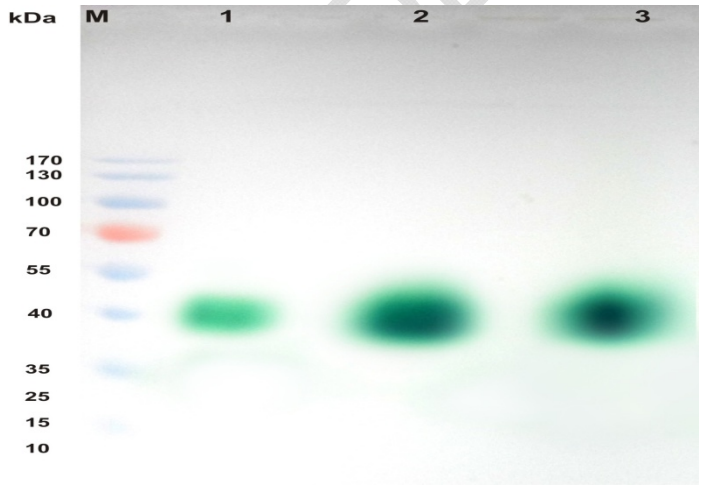

Plate 2. Molecular mass of laccase of Trametes sp. G31 on N-PAGE
M=Marker (Prestained protein ladder, 10 - 170 kDa, Invitrogen); 1=crude laccase; 2=ammonium sulphate precipitated

laccase; 3=dialysed laccase.

3.4. Decolourization of synthetic dyes

Several studies have demonstrated the ability of fungal isolates including *Trametes* sp. to decolourise dyes. In this study, the ability of crude laccase from *Trametes* sp. isolate G31 to decolourise structurally different synthetic dyes was determined (Fig. 11). The study showed that 40% of Phenol red was decolourised after 1 hour of treatment with 1000 U/mL of crude laccase. In that, the crude laccase oxidized Phenol red in absence of any mediator was uncommon characteristic because Phenol red comparatively posses a high oxidation potential [51]. However, the rate of decolourisation declined suddenly to 8.4% and then 20% at 24 and 48 hours respectively. This was because enzymatic degradation of dyes is a stepwise process, which involves 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 due to polymerization of dye fragments and a resultant darkening of solutions [54].

The enzyme was more efficient in decolourising RBBR with 45% and 93% after 1 hour and 24 hours respectively. However, another study reported 74.4% and 76% decolourization of RBBR for 6 and 12 days using laccase produced on Citric pulp and Coffe husks respectively by *Lentinus crinitus* [55]. Congo red, Methylene blue and Crystal violet successfully achieved 51%, 53% and 60% decolourisation at 120, 48 and 72 hours respectively. Methyl red and Malachite green attained 42% and 32%decolourisation at 72 and 48 hours respectively. Another study showed that crude laccase of *Pichia manshurica* DW2 decolourized 84% and 76% of Malachite green and Methyl red respectively at 96 hours at 0.1 g/L [19]. 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 [8]. 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* [37].

In this study, crude laccase from *Trametes* sp. isolate G31 decolourised 93% of Remazol Brilliant Blue R in 1 hour but also achieved 51% successful decolourisation of Congo red after 120 hours instead of 13 days [37]. 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 [37]. 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 [53]. However, this study showed that crude laccase from *Trametes* sp. isolate G31 attained 51 and 60 % decolourisation of Congo red and Methyl orange without the use of enzyme mediators. Moreover, this result contrast with those of laccase from *P. radiat*a strain BP-11-2, which did not decolourise Congo red or Methyl orange [53].

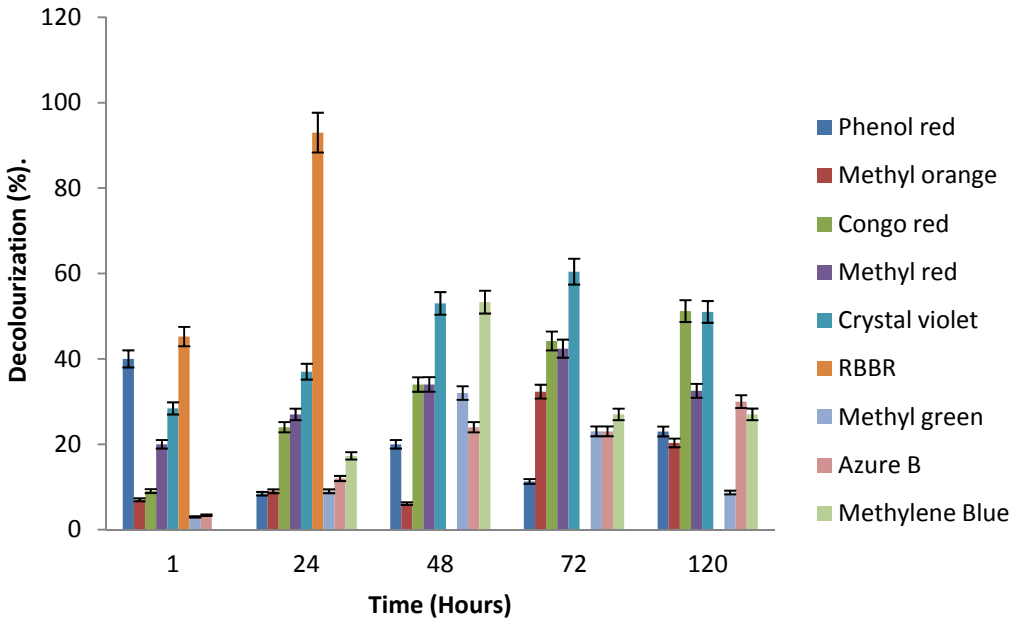

Figure 11. Percentage decolourization of different synthetic dyes using 1000 U/mL laccase from *Trametes* sp G31.

4. CONCLUSION

This study established that *Trametes* sp. G31 produced high titers of laccase using various methods of optimisation including cultural and nutritional factors. pH, temperature and duration of incubation had the greatest effects on laccase production. Among the sources of carbon tested, sucrose induced higher laccase production while ammonium sulphate was the best source of nitrogen. Effects of metallic ions showed higher stimulatory influence of Cu^{2+} and Ca^{2+}. However, the stimulatory effect of ABTS and other activators on laccase production was not significantly different. The choice of an agroindustrial waste such as sawdust provided a cheap alternative substrate with eco-friendly benefits and high yield for laccase production in SSB. The partial purification of crude laccase showed a specific activity of 5008, purification factor of 3.85% yield and molecular mass of 40 kDa on N-PAGE using ABTS. The crude laccase demonstrated great potential to decolourize synthetic dyes of diverse structure. RBBR (93%), CV (60%), Methylene blue (48%) CR (51%) and Phenol red (40%) attained different rates of decolourization due to their chemical structures and the enzyme systems. The capacity to decolourize Phenol red and other dyes without enzyme mediators showed strong oxidation potentials of the enzyme and a suitable tool for many industrial processes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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