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Production of Fungal Laccase Using Orange Peelings as Substrate by Submerged Static Fermentation

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Authors' contributions

This work was carried out in collaboration between both authors. Author FSI designed the study, provided technical expertise, performed the statistical analysis and proofread the manuscript. Author EFA wrote the protocol, managed the analyses of the study and wrote the first draft of the manuscript. Both authors managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Laccases are diphenol oxidases that have numerous applications in biotechnological processes. In this work, the production of fungal laccase using organic and inorganic feed substrates in submerged static fermentation was investigated.

Study Design: One-factor-at-a-time strategy was adopted to optimize the cultural parameters for enhanced laccase production.

Place and Duration of Study: Department of Microbiology, Faculty of Science, University of Port Harcourt, Nigeria, between October 2014 and November 2015.

Methodology: A total of nine fungal isolates were obtained from wood decaying sites of University of Port Harcourt forest areas and subjected to laccase screening with 2,2-Azinobis-3-ethyl(benzthiazoline-6-sulphonate) (ABTS). The influence of medium components using the basal medium at pH 5.0 as base was evaluated and these cultural parameters include carbon sources (glycerol, rice bran, glucose and ground orange peelings), nitrogen sources (yeast extract,

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potassium nitrate, peptone and ammonium chloride), metal ions (copper sulfate and manganese sulfate) and inducer compounds (ABTS, Tween 80 and soya oil). Time course study was conducted with the unoptimized and optimized cultural medium.

Results: Out of nine cultures tested, seven were found to be laccase-positive with isolates CF-1 and CF-2 being the best potential cultures. Isolate CF-1 which had the highest laccase activity was identified as *Pleurotus ostreatus* and was chosen for further studies. Ground orange peelings (0.1% w/v) and NH₄Cl (0.1% w/v) were the most suitable carbon and nitrogen source for laccase production by the fungus. Maximum laccase production was obtained with Cu²⁺ at a concentration of 0.05%w/v among other metal ions. Soya oil at concentration of 0.05% (v/v) was the best inducer of the enzyme. The highest laccase production was achieved at an Initial pH 4.5. Under optimal culture medium, the maximum laccase activity was determined to be 7.21 U ml⁻¹ on the 7th day of cultivation; which was approximately three times higher than that in basal medium (2.5 U ml⁻¹). The results obtained indicate that the extracellular laccase production is dependent on various cultural parameters.

Conclusion: One-factor-at-a-time strategy adopted in this study proved that the optimum conditions enhanced laccase production by three folds using orange peelings. The results obtained are very interesting since orange peelings are common agricultural wastes in several countries and imply that their re-utilization in the production of enzymes would help solve pollution problems caused by their improper disposal. In addition, *Pleurotus ostreatus* having shown promise for laccase production using low-cost lignocellulosic substrates could be suggested as a prospective candidate for higher laccase production for several biotechnological applications.

Keywords: Laccase; ABTS; *Pleurotus ostreatus*; submerged fermentation; static; orange peels.

1. INTRODUCTION

Laccases (1,4-benzenediol: oxygen oxidoreductase; EC 1.10.3.2) are multi-copper polyphenol oxidases that catalyse the one electron enzymatic oxidation of a wide variety of both organic and inorganic substrates with the concurrent four electron reduction of oxygen to two water molecules [1,2]. They are among the important enzymes that have attracted tremendous attention in recent years due to their important applications in different industries [3]. Along with the plant ascorbate oxidases and the mammalian plasma proteins, they belong to a group of enzymes called the Blue Copper proteins due to their characteristic four (4) catalytic copper atoms [4]. As such, the laccase molecule is a dimeric or tetrameric glycoprotein with four copper atoms distributed in three (3) redox sites with one copper atom placed at the Type 1 site, where reducing substrates bind. The other three copper atoms are clustered in the Type 2/Type 3 trinuclear site, where the reduction of molecular oxygen and the eventual release of water take place [5,6].

The broad substrate specificity of laccases holds promise to use them for biotechnological purposes, such as biomechanical pulping, bleaching of pulp, degradation of dye, and transformation and detoxification of xenobiotic and other aromatic compounds [7]. Numerous

studies have shown the potential of fungal phenol oxidases as a biological alternative for chemical oxidative process like in pulp delignification, textile industries, food industries, organic synthesis, pharmaceutical sector, bioremediation and nanobiotechnology [8]. Laccase production by white-rot fungi is often associated with their lignin degrading abilities, although laccases play a role in some other functions such as sporulation, pigment production and fruiting body development [1]. Laccases have been isolated from many plants, fungi, actinomycetes and bacteria [9]. Most of the laccase studies are of fungal origin especially from white rot fungi [10-17]. *Pleurotus ostreatus* is one of the most extensively studied white-rot fungi as potential laccase producers for its exceptional ligninolytic properties; fast growth and easy handling under field conditions [3]. Screening of a large number of white rot fungi is necessary to select strains that are able to produce high titres of laccases with novel characteristics. Production of laccase by microorganisms has been carried out in both submerged and solid state fermentation [18]. Under the submerged fermentation, static or agitated conditions could be used for the cultivation of the organism for enzyme production. Previous reports indicated that high titre of laccase has been obtained with static conditions than agitated fermentation processes [19].

The application of laccases in biotechnological processes requires the production of high amounts of enzyme at low costs, as such the current focus of laccase research is oriented towards the search for efficient production systems. In addition, production of laccase is affected by many typical fermentation conditions such as medium composition, carbon and nitrogen ratio, pH, temperature and aeration rate [20]. In particular, the selection of appropriate medium components and use of inexpensive sources (industrial and agricultural wastes) for laccase production is crucial in the development of an efficient and economic process. According to Cilerdzic et al. [21] agricultural and industrial expansion has led to the production of large amounts of agricultural, forest, industrial and domestic wastes which invariably could pose serious environmental pollution. This informed the decision to evaluate rice bran and orange peel for their potential as sole carbon source for laccase production by fungi. Fungal laccase production is highly regulated by the media composition and hence medium optimization has become one of the main methods to enhance laccase production [22]. Therefore, this present study was aimed at isolation, screening of potential laccase producing fungi and assessing the ability of selected agro-waste residues for laccase production as well as optimize the laccase production by *P. ostreatus* under submerged static conditions using One-Factor-at-a-Time methodology.

2. MATERIALS AND METHODS

Soil samples were collected from different wood decaying sites in forest areas of University of Port Harcourt, Choba, Rivers State at 5-10cm depth into sterile nylon bags.

2.1 Isolation and Screening of Fungal Strain

One gram of dried soil sample was diluted ten times in 9ml sterile distilled water by the method of Gochev and Krastanov [23] before plating on Potato Dextrose Agar, Soil Extract Agar and Malt Extract Agar, all fortified with 0.05 mg/ml Chloramphenicol and incubated at 30°C for 7 days. Isolates were subjected to laccase screening on Malt Extract Agar fortified with 0.25 mg/ml ABTS (2,2-Azinobis-3-ethyl(benzthiazoline-6-sulphonic acid)) as an indicator [24,25]. Isolates with the highest

laccase activity were chosen and identified microscopically on a wet mount where the shape, size and arrangement of the conidiophores and conidiospores were studied. The morphological examinations were observed on PDA and Glucose-MEA with reference to the standards of Samuels et al. [26] and subsequently maintained as slants on a Glucose-MEA. These slants were sub-cultured every two (2) weeks.

2.2 Basal Medium Composition

The medium of Gochev and Krastanov [23] was used as basal medium to assess the ability of the fungus to produce laccase in liquid medium. The basal medium consisted of 10 g Glucose, 1 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.005 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g $(\text{NH}_4)_2\text{SO}_4$, 0.005 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g KCl, 0.5 g peptone per litre. Medium was adjusted to pH 5.0. The soil-derived basidiomycete *Pleurotus ostreatus* was used as the laccase source. Fungal spore suspension prepared by homogenizing the 96 h old freshly grown slants with 2 ml sterile distilled water for 5s was used as the inoculum. One millilitre (1 ml) inoculum (2%v/v) was introduced into 250 ml Erlenmeyer flasks containing 50 ml basal medium and incubated at 30°C for 5 days under submerged static condition. After every 24 h of cultivation, samples were prepared by filtration using Whatman No. 1 filter paper. The obtained cell free supernatant was used for the determination of laccase activity.

2.3 Effect of Carbon Sources on Laccase Production

Various carbon sources such as glycerol, rice bran and orange peel at 0.5% (w/v) were separately added in place of glucose as sole carbon sources in the basal culture medium of the fungus for optimization of the carbon source [24].

2.4 Effect of Nitrogen Sources on Laccase Production

Different concentrations of organic and inorganic nitrogen sources were examined for maximum production of laccase. Peptone in the basal medium containing the best carbon source was replaced with varying concentration (0.1-1.0% (w/v) of potassium nitrate (KNO_3), ammonium chloride (NH_4Cl) and yeast extract using the method of D'Souza-Ticlo et al. [27].

2.5 Effect of Copper and Manganese Ions on Laccase Production

Copper ion (Cu^{2+}) and Manganese ion (Mn^{2+}) at varying concentrations (0.05-0.1%w/v) each were incorporated into the fermentation medium containing the best concentrations of carbon and nitrogen sources, according to the method of Liu et al. [25].

2.6 Effect of Inducers on Laccase Production

Soya oil, Tween 80 and ABTS as inducer compounds were incorporated into the fermentation medium containing the best concentrations of the carbon and nitrogen sources, and metal ion according to the method of Osma et al. [28].

2.7 Effect of Initial pH on Laccase Production

The effect of pH on laccase production was studied by adjusting the pH of the medium between pH 4.0 to 8.0 with 0.1N NaOH or 0.1N HCl before sterilization [29]. Fermentation was carried out at conditions earlier mentioned using orange peels and ammonium chloride as carbon and nitrogen sources, respectively.

2.8 Time Course Study of Laccase Production

The influence of time course on the production of laccase was examined with the optimized medium parameters which comprised of 0.1% (w/v) orange peelings, 0.1% (w/v) NH_4Cl , 0.05% (w/v) CuSO_4 and 0.5% (v/v) soya oil and initial pH 4.5 that favoured high laccase production. Fermentation was carried out by incubating the fungus at 30°C for different time periods of nine days and after each 24 h of incubation, the final pH, biomass yield and laccase activity were determined using standard methods.

2.9 Analytical Methods

2.9.1 Enzyme activity

Laccase activity was estimated spectrophotometrically at 420 nm as described by Birhanli et al. [13] with modifications using ABTS (2,2-Azinobis-3-ethyl(benzthiazoline-6-sulphonate)) as a substrate. The assay mixture contained 1.50 ml Na-Acetate buffer (0.1 M) pH

5.0, 1.00 ml cell-free culture filtrate and 0.50 ml ABTS (0.1 M). Oxidation of ABTS was monitored by an absorbance increase at 420 nm at 30°C. The blank contained all the assay constituents except the active enzyme while buffer or heat inactivated enzyme was used in its place. Enzyme activity was expressed as U/ml. One activity unit was defined as the amount of enzyme producing a 0.001 increase in the optical density per ml per minute of the culture filtrate (U/ml) under the standard reaction condition [27]. All values were the means of three replicates, with standard deviation of the means shown as \pm values.

2.9.2 Fungal identification

The isolated fungi were identified on the basis of morphological (macro and micro) and physiological characteristics as described by Aneja [30]. The following morphological characteristics were examined and used to identify the fungus: colony growth, presence or absence of aerial mycelium, colony colour, presence of wrinkles and furrows, pigment production among others [31]. Also the spore structure of the fungal isolates was stained by cotton blue and Lactophenol [32] observed under compound microscope with reference to standard atlas and manuals.

2.9.3 Biomass evaluation

The fungal biomass was recovered after fermentation by filtration using a pre-weighed Whatman No. 1 filter paper. Thereafter, the mycelial mat in the pre-weighed Whatman No. 1 filter paper was dried at 70°C until a constant weight is obtained. The difference in weight between the pre-weighed filter paper and mycelial mat-bearing Whatman No. 1 filter paper represented the biomass of fungal mat and expressed in gram fungal dry weight per 50 ml of medium (g/50 ml) [28].

2.10 Statistical Analysis

The data obtained in this study was subjected to statistical analysis using single factor analysis of variance (ANOVA) at a significance level of $p < 0.05$.

3. RESULTS AND DISCUSSION

This study was aimed at isolation and screening of potent laccase producing fungi as well as

evaluation of various physicochemical parameters to optimize laccase production under submerged static conditions. Among the 30 samples collected, 7 strains out of 9 fungal isolates were selected as positive organisms which formed varied degrees of a reddish-brown coloured zone around the colonies. Based on the reddish brown zones, seven strains were designated as CF-1 to CF-7 (Table 1). Strain CF-1 having the highest diameter of decolourized zone was selected for further studies. The result obtained is in agreement with the report of Kiiskinen et al. [33] for the isolation of laccase producing fungus. Morphological examinations presented them as cottony-white eccentric colonies on MEA covering plate, with a light brown reverse side. The isolates were non-sporulating with abundant clamp connections forming in mycelia, a microscopical characteristic of basidiomycetes. This isolate was tentatively identified as *Pleurotus ostreatus*.

3.1 Laccase Production Using Basal Medium

Fig. 1 shows laccase production by *P. ostreatus* using the basal medium in a 5 day time course study with unoptimized medium. Results obtained show that laccase secretion by this

fungus commenced after 24 h of cultivation. The result indicated that maximum laccase activity (2.5 U ml^{-1}) occurred on the 4th day of incubation at pH 5.6. Also, the highest growth of the fungus was obtained on day 4. Further incubation beyond the fourth day resulted to decrease in laccase activity and growth of the fungus. It could be suggested that the low pH and low glucose (from the basal medium) may have caused an early but slow laccase secretion with a corresponding slow biomass increase. This result is consistent with earlier reports of Galhaup et al. [34].

Table 1. Screening of laccase-producing fungi by plate tests using the indicator compound ABTS

Isolates	Fungi	Diameter of decolourized zones (mm)
CF-1	<i>Pleurotus sp.</i>	6.0
CF-2	<i>Pleurotus sp.</i>	5.0
CF-3	<i>Rhizopus sp.</i>	1.3
CF-4	<i>Aspergillus sp.</i>	0.5
CF-5	<i>Penicillium sp.</i>	2.7
CF-6	<i>Rhizopus sp.</i>	1.5
CF-7	<i>Penicillium sp.</i>	2.9

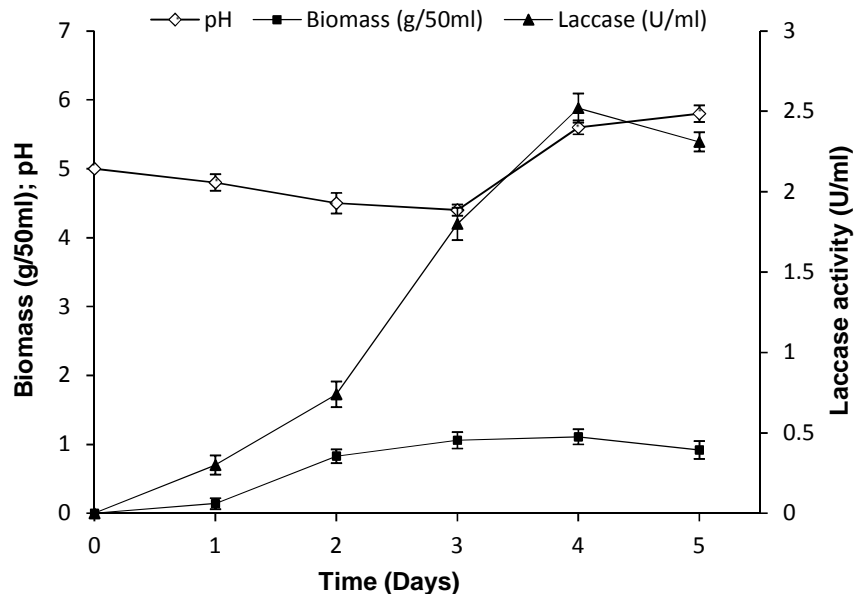


Fig. 1. A 5-day time course study of laccase production with basal medium (unoptimized medium) by *P. ostreatus*

3.2 Effect of Carbon Sources on Laccase Production

A comparison of the effect of some carbon substrates (glucose, glycerol, rice bran and orange peelings) on laccase production was investigated. Fig. 2 shows the effect of carbon sources on laccase production. Maximum laccase production was obtained with orange peelings (4.60 U/ml) followed by rice bran (3.9 U/ml). Glucose showed the least laccase activity (2.50 U/ml). This result indicates that ground orange peelings had a higher inductive capacity in liquid medium than rice bran, glucose and glycerol. This result also revealed that agricultural waste material used in this study had more stimulatory effect on the production of laccase by the fungus than other sugars and indicated the ability of the fungus to utilize inexpensive and easily available substrate for laccase production. This assertion is in agreement with previous observations that agricultural waste residues are good carbon sources for low cost laccase production [18,21]. According to Osma et al. [28] this is likely related with their contents in water-soluble aromatic compounds (flavones and flavonoids) which is capable of inducing or stimulating the biosynthesis of ligninolytic enzymes. Moreso, the cellulose of orange peelings may have acted as an activator of laccase activity [35]. Patel and Gupte [36] have reported the use of lignocellulosic substrates such as wheat straw as carbon source for the production of laccase. Chen et al. [9] reported the use of bagasse, corncob and sawdust as carbon source to produce laccase from *T. fusca* BCRC 19214. The result of this study is also in agreement with previous reports on *P. ostreatus* where ground mandarin peelings were used to obtain high laccase productivity [28]. Sawdust has been reported as the best carbon source for laccase production by *Coriolopsis gallica* and *Ganoderma lucidum*, respectively [18,21]. This result seems very interesting since orange peelings are common agricultural wastes in several countries including Nigeria and implies that their re-utilization would help solve pollution problems caused by their inefficient disposal.

Highest biomass yield was observed in the medium containing glucose (0.53 g/50 ml) followed by that containing glycerol (0.32 g/50 ml) (Fig. 2). The medium containing rice bran showed the least biomass production (0.185 g/50

ml) within the 96h culture period. The result of this investigation showed that the orange peelings and rice bran that had high stimulatory ability for laccase production were poor in supporting the growth of the fungus while glucose encouraged growth but had poor laccase stimulation. D'souza-Ticlo et al. [27] suggests that higher amounts of biomass build-up in the medium containing glucose probably were merely a function of the assimilability of the carbon. This could be justified by the low trend of biomass concentration in the other media containing a little more complex sugar as carbon source.

Fig. 3 shows the effect of orange peelings concentration on laccase production. The result reveals that the medium containing 0.1% (w/v) orange peelings exhibited a markedly high laccase activity (7.44 U/ml) followed by that with 0.3% (w/v) (4.78 U/ml). The least laccase activity was observed in the medium containing 0.7% (w/v) orange peelings (1.96 U/ml). D'souza-Ticlo et al. [27] reported a delay in laccase production in the presence of high concentrations of the carbon source as previously observed in *Trametes versicolor*. Thus, suggesting that lower concentrations of not-very-easily assimilable carbon sources may overcome the time delay in laccase activity by allowing for constitutive as well as inducible laccase production as incubation time progresses. The result agreed with those previously reported by Osma et al. [28] and Stajic et al. [37]. Hou et al. [38] reported maximum laccase activity with cellobiose as a carbon source after a 12-day static cultivation, noting that the fungus was able to produce laccase on glucose, glycerol, cellulose and starch but with varied activities. Wang et al. [39] reported increased laccase expression from *Monotospira sp.*, under carbon-limited cultures and suggested that better enzyme production may be achieved as far as the conditions in which these fungi grow is concerned. However, other previous reports have indicated laccase activity in media with glucose [38] and sucrose [39] as carbon sources. Lee et al. [40], suggests that excessive concentrations of glucose are inhibitory to laccase production in various fungal strains. Also, an excess of sucrose also reduced the production of laccase by blocking its induction and only allowed constitutive production of the enzyme. Use of polymeric substrates like cellulose was able to minimize this problem [41].

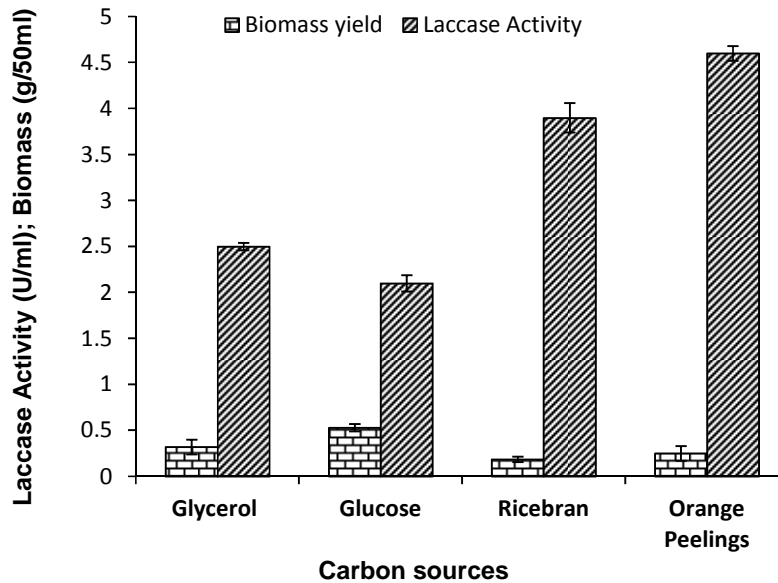


Fig. 2. Effect of different carbon sources on laccase production and biomass yield after 96h cultivation

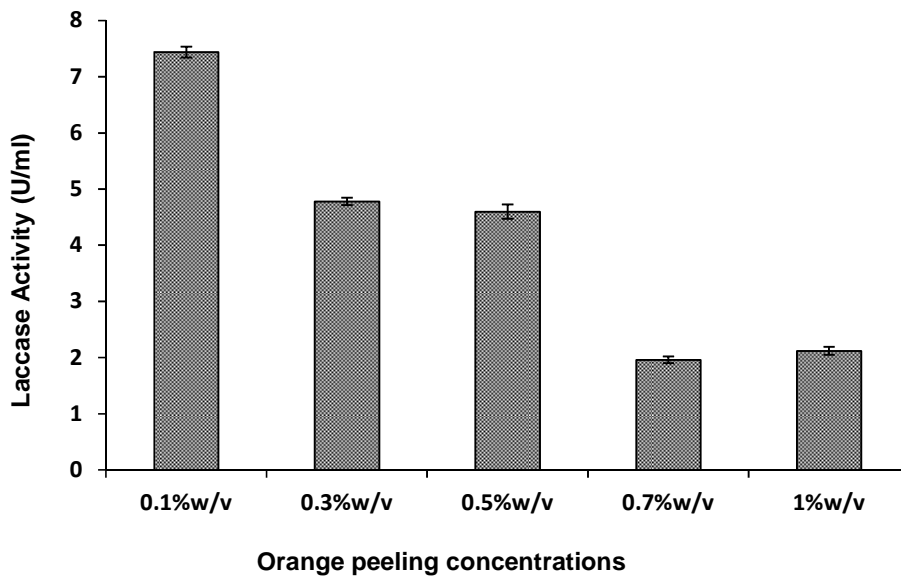


Fig. 3. Effect of orange peeling concentrations (0.1-1.0%w/v) on laccase production after 96h cultivation

3.3 Effect of Nitrogen Sources on Laccase Production

The influence of various nitrogen sources on laccase production by the fungus was evaluated. The effect of nitrogen sources on laccase production is depicted in Fig. 4. The result indicated that the medium with NH_4Cl exhibited highest laccase production (6.95 U/ml) after 96 h. This was followed closely by that containing

yeast extract (6.20 U/ml) while KNO_3 had the least laccase production (1.90 U/ml). Lignolytic enzyme production has been observed in both nitrogen-sufficient and nitrogen-deficient media using different organisms [36]. However, the influence of nitrogen source on laccase secretion by different microorganisms seems to be controversial [42]. Patel and Gupte [36] observed that the presence of both organic and inorganic nitrogen sources in the medium favoured higher

laccase production by *T. giganteum* but did not encourage growth. Dong et al. [43] have reported enhanced laccase production in the presence of tryptone and peptone. Yeast extract has been reported to be a suitable nitrogen source for laccase production [44]. Moreso, studies have shown that both the nature and concentration of nitrogen sources are powerful nutrition factors regulating ligninolytic enzyme production by wood-rotting basidiomycetes [34]. Monteiro and De Carvalho [45] had reported high laccase activity with *Trametes versicolor* using low carbon to nitrogen ratio. However, Buswell et al. [46] reported a higher laccase production at high carbon to nitrogen ratio. Compared to inorganic N sources, Hou et al. [38], reported increased laccase activity of *P. ostreatus* strain 32, with organic N sources as the only N-source.

The biomass yield with regard to different nitrogen sources is also presented in Fig. 4. The result revealed that the medium containing yeast extract favoured the highest biomass yield (1.21 g/50 ml), followed by peptone (0.84 g/50 ml) whereas at NH_4Cl gave the least biomass yield (0.49 g/50 ml). It has been suggested that yeast extract and peptone as organic sources may have served as both nitrogen source and as a readily available source of carbon [27].

The result of the effect of different concentrations (0.1-1.0%) of the NH_4Cl on laccase production is presented in Fig. 5. Highest laccase activity (6.50 U/ml) was obtained with a concentration of 0.1% (w/v) followed by 0.3% (w/v) (4.61 U/ml) while at a concentration of 1.0% (w/v), the least laccase activity (2.23 U/ml) was observed. Generally, the result showed that increasing the concentrations of NH_4Cl did not favour laccase production. The influence of NH_4Cl on biomass yield was negative throughout and its maximum negative impact coincided with maximum laccase production by the 4th day. This might be a synergistic effect of orange peelings and ammonium chloride, indicating that the concentration of nitrogen was critical for laccase production. Shradha et al. [47] suggested that laccase production is often triggered by nitrogen depletion, although, it was also found that in some strains, nitrogen had no effect on enzyme production. Keyser et al. [48] opined that the low nitrogen content of the synthetic culture or fermentation medium mimics the nitrogen depletion that occurs when the fungus reaches its idiophase, thereby triggering laccase production.

3.4 Effect of Copper and Manganese Ions on Laccase Production

Result of the effect of copper and manganese ions on laccase production is shown in Fig. 6. This result indicates that the medium supplemented with Cu^{2+} exhibited a higher laccase production (6.99 U/ml) than the Mn^{2+} (3.98 U/ml). According to Wang et al. [49], carbon sources and copper ions are the two most critical factors in improving or stimulating laccase production. Different studies have shown that laccase production is regulated by metal ions such as Cu^{2+} and Fe^{2+} by gene expression induction or through translational or post-translational regulation [50]. Our result is consistent with Patel and Gupte [36] who reported maximum laccase production with copper sulphate. Moreso, Manavalan et al. [51] reported significant increase in laccase production (1.5 U/ml) by *Ganoderma lucidum* when the culture was supplemented with 0.4 mM CuSO_4 . Maximum laccase production (34.6 U/ml) was obtained when 0.25 mM CuSO_4 was added into the medium for *Pycnoporus cinnabarinus* [52]. According to Passarini et al. [22], *Nigrospora* sp. CBMAI 1328 produced highest level of laccase (25.2 U/L) in the presence of 5 μM CuSO_4 after 120 h of incubation. In contrast with our finding, the authors also reported that *Arthopyrenia* sp. CBMI 1330 presented highest laccase activity without copper sulphate in the medium. The positive effect of Cu^{2+} addition on the production of laccase by *Coriolopsis gallica* was reported by Daassi et al. [18].

On biomass yield, manganese ion (Mn^{2+}) encouraged more fungal growth (1.81 g/50 ml) than the copper ion (Cu^{2+}) (1.32 g/50 ml) after a 96h of incubation. D'souza-Ticlo et al. [27] opined that Cu^{2+} can have a negative coefficient with other media components on biomass accumulation. Our result is in contrast with the work of Passarini et al. [22] who reported high biomass production in the presence of copper sulphate for *Nigrospora* sp. CBMAI 1328. Copper ion as a micronutrient has a key role as a metal activator; induces laccase transcription; and plays an important role in laccase production [53]. Overall, laccase production is reported to be induced in the presence of Cu^{2+} [54]. A possible explanation for this stimulatory effect of copper on laccase biosynthesis could be a role for the enzyme activity in terms of a defense mechanism against oxidative stress, with laccase involved in the synthesis of pigments to prevent the uptake of metals [55]. Furthermore, the promoter regions

of laccase genes have been shown to contain various recognition sites specific for heavy metals which when bond to, induce laccase production [56].

Furthermore, various concentrations of copper in the range of 0.01-0.1% (w/v) were studied. Fig. 7 shows the effect of copper ion concentration on laccase production. Maximum production of laccase (9.33 U/ml) was obtained at 0.05% (w/v) copper concentration. Further increment of copper concentrations beyond 0.05% (w/v) resulted in corresponding decrease in laccase production. Passarini et al. [22] and Liu et al. [25]

opined that cu^{2+} requirements by microorganisms are usually satisfied by very low concentrations of 1-10 μ M but toxic to fungal metabolism at higher concentrations. The trend of our result on effect of copper concentration on laccase production may be because of insinuation that higher concentration of copper is inhibitory to fungal growth [44]. Similar result has been reported from *Tricholoma giganteum* [36]. Our result is consistent with Manavalan et al. [51] and Mann et al. [57] who reported that inclusion of 0.75 and 0.4 mM copper significantly induced laccase production in *Cerrena consors* and *Ganoderma lucidum*, respectively.

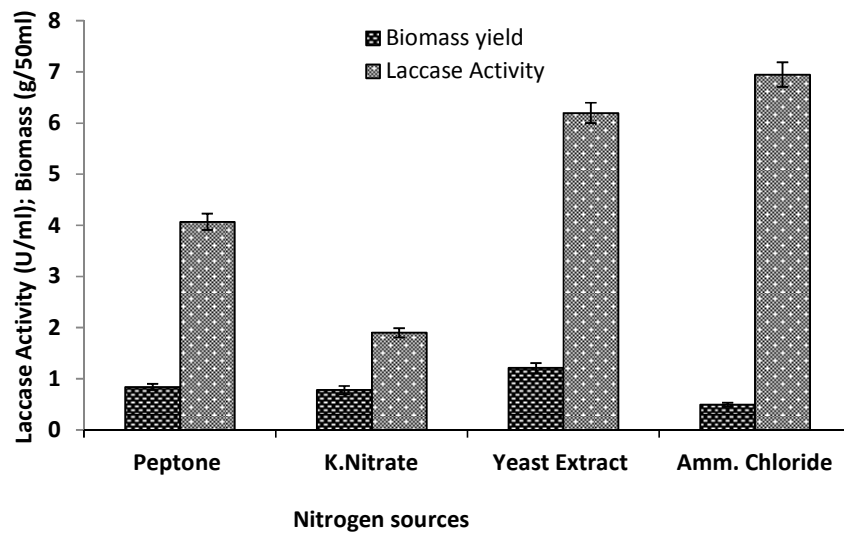


Fig. 4. Effect of different nitrogen sources on laccase production and biomass yield after 96 h cultivation

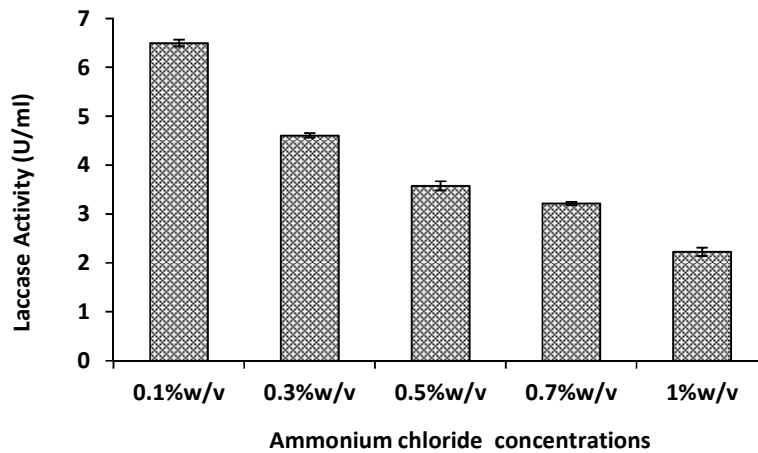


Fig. 5. Effect of ammonium chloride concentrations (0.1 - 1.0% w/v) on laccase production

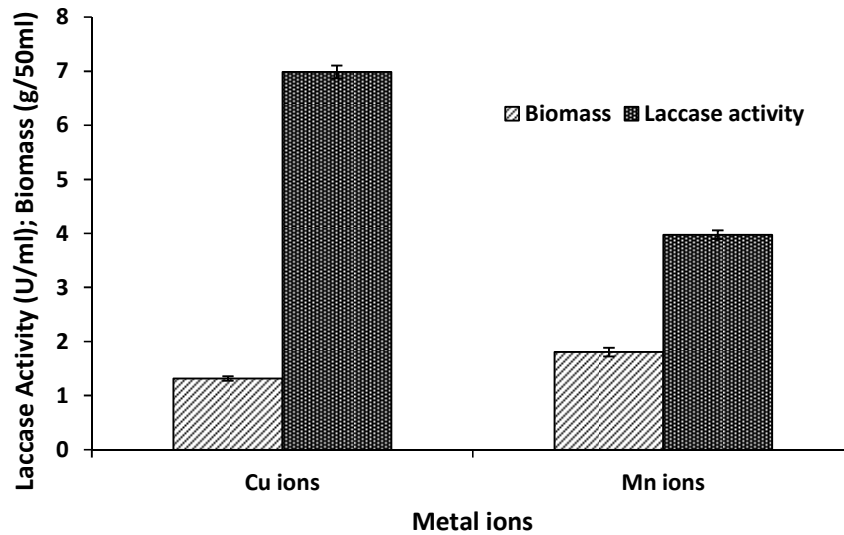


Fig. 6. Effect of metal ions on laccase production and biomass yield after 96 h cultivation

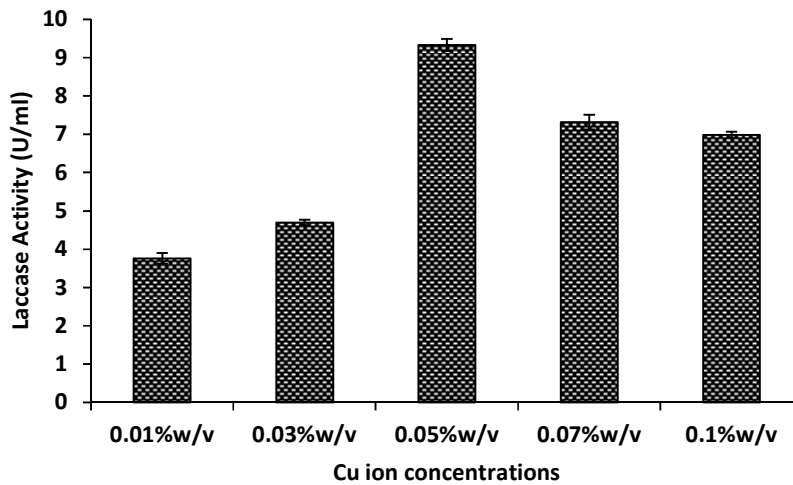


Fig. 7. Effect of Cu ion concentrations (0.01-0.1%w/v) on laccase production after 96h cultivation

The role of copper in the enhancement of laccase activity has been well demonstrated in both fungi and bacteria [58,59]. However, Chen et al. [9] reported contrary results from *Thermobifida fusca* as well as a reduced laccase activity by metal ions of Hg, and is similar with the results from *Streptomyces psammoticus* [60], *Trametes hirsute* [61] and *Paraconiothyrium variabile* [62]. In an orthogonal matrix study showing effects of copper with a new strain of white rot fungus *WR-1* isolated from tree bark, laccase production was considerably enhanced

to 410 U/ml by incorporation of 0.5 to 1 mM copper in the optimized medium [44]. According to the report a significant reduction in cell growth with substantial decrease in laccase activity was observed at 2 mM concentration of copper. Infact, further increase of copper to 5mM led to almost negligible cell growth and very small laccase activity. Dhakar and Pandey [63] also reported a 2-fold increase in laccase production with maximum being at 0.4 mM concentration and further increment in CuSO_4 (up to 1.0 mM) resulted in decline of laccase production. CuSO_4

is a constituent of the catalytic centre of the laccase; hence it is important for the synthesis of the respective enzyme [64].

In addition, reports suggest that the effect of Cu^{2+} on laccase production might be related to transcriptional regulation of laccase genes, as well as positive adjustment of laccase activity and stability [29]. In this present study, the media containing Cu^{2+} concentrations beyond 0.05% (w/v) showed a significant decrease in laccase production and fungal growth. This may be attributed to the inhibitory effect of Cu^{2+} at higher concentrations as equally observed by Passarini et al. [22]. Our result is in agreement with the reports of Galhaup et al. [34] and Stajic et al. [37] who separately reported that the addition of CuSO_4 in various concentrations (1-10 mM) stimulated laccase production in *T. pubescens*, *P. ostreatus* HA1493, *P. ostreatus* HA1494 and *P. eryngii*. Cordi et al. [29] observed that the inclusion of 0.007-0.1 mM copper sulphate during into the growth medium of *Trametes versicolor* enhanced high laccase activity. This result showed that the addition of CuSO_4 at very low concentrations favours production of laccase by *P. ostreatus* but slows biomass accumulation. Thurston [1] affirmed that metals are required by fungi for their normal growth and laccase biosynthesis. According to the author, one molecule of laccase requires four copper atoms for its full function.

3.5 Effect of Inducer Compounds on Laccase Production

Laccase production has been found to be highly dependent on the conditions for cultivation and nutritive media composition [65,66]. In this study, addition of these supplements to the media improved laccase production in varying degrees. Zheng and Obbard [67], opined that these inducers, being surfactants especially Tween 80, can increase the bioavailability of less soluble substrates for the fungi and stimulate the growth of the fungal spores. The effect of inducer compounds on laccase production is shown in Fig. 8. Soya oil exhibited a markedly higher laccase activity (5.89 U/ml) followed by Tween 80. ABTS showed the least laccase activity (1.64 U/ml) within the incubation period. This result is in agreement with Osma et al. [28] who observed the influences of ABTS, Tween 20, soya oil, and Malachite green on laccase production, with soya oil yielding a markedly high laccase activity than the other inducers. Interestingly, the above-

mentioned compounds are non-toxic contrary to those commonly used to stimulate laccase synthesis [44]. Patel and Gupte [36] also reported the increase in yield of laccase after the addition of Tween 80. Similar results have been reported in other studies [68,69]. Soya oil promoted highest biomass yield (1.11 g/50 ml) followed by Tween 80 (0.98 g/50 ml). The least biomass was observed in the medium with ABTS (0.72 g/50 ml). Reports suggest that the positive impact of soya oil on enzyme production may be due to its surfactant property which emulsifies the fungal membrane thereby aiding the release of cell membrane-associated laccases as well as in the secretion of the normal extracellular laccases [70].

The effect of various concentrations of soya oil on laccase secretion is depicted in Fig. 9. The different concentrations elicited varied levels of laccase secretion. Maximum laccase production was obtained at 0.5% (v/v) soya oil concentration after which further increase in soya oil concentration led to decrease in laccase production. The lowest laccase production was noticed at 0.1% (v/v). Liu et al. [25] suggested an antagonistic effect of inducers on CuSO_4 by reducing laccase production. According to the report, the reason is not clear, but may be due to the poisoning of these inducers for cell growth as well as laccase production under selected concentrations. Cserhati [70] suggested that the role of soya oil may be due to either of the following reasons or a combination thereof; (a) The surfactant property of soya oil which emulsifies the fungal membrane aiding in the release of cell membrane-associated laccases as well as in the secretion of the normal extracellular laccases. (b) The decrease in amount of evaporation of moisture due to the presence of soya oil, which would otherwise have led to the concentration of medium components during prolonged incubation periods and thus, either increase adverse interactions between compounds or the precipitation of critical components. Wang et al. [39] opined that surfactants increase the activity of the already secreted enzyme, although the specific mechanism by which they enhance extracellular enzyme production in filamentous fungi has not been elucidated.

3.6 Effect of Initial pH on Laccase Production

The hydrogen ion concentration (pH) is one of the important parameters in fungal cultivation

and tolerance to a wider range is indicative of the ecological resilience possessed by the fungus [63]. The optimum pH value for laccase production varies depending on the substrates employed, although many reports have suggested a bell-shaped profile for laccase activity [47]. The optimal activity is mainly obtained in the pH range of 3.0-5.0 [36]. The effect of pH of the culture medium was varied from 4.0 to 8.0. In this present study, the maximal laccase production was obtained at pH 4.5 (7.09 U/ml) (Fig. 10). However, further rise in pH showed no increase in the production of the laccase. This may be attributed to the poor mycelial growth at an increased pH which may hinder laccase production. Similar result has been reported by Diaz et al. [71] with maximum enzyme production at pH 4.5. Our result is consistent with the reports of Strong et al. [72] and Patel et al. [3] with optimum laccase production at pH 4.5. The result obtained in the current study differs with the observation of Janusz et al. [73] who reported pH 7.5 as optimum for laccase production by *Rhizoctonia praticola*. Our result is also at variance with pH 3 reported by Kocyigit et al. [74] as the optimum pH for laccase production from *Tramete strogii*. The optimum pH for L1 (isozyme of laccase) was 4.0 whereas the optimum pH for L2 was 5.0. Laccases extracted from *Trametes versicolor* by Han et al. [75] showed high enzyme activity at a broad pH range with the optimal being 3.0. Contrary results have been reported by Patel and Gupte [36] and Ravikumar et al. [76] with maximum enzyme production at pH 5.0. In a recent work by Daassi et al. [18], maximum laccase production by *Corioloopsis gallica* was obtained at pH 5.0. In addition, Chhaya and Gupte [77] reported maximum laccase production at pH 5.0 using one-factor-at-a-time methodology under solid state fermentation. However, most studies show that pH between 4.5 and 6.0 is suitable for enzyme production [1,78,79]. Maximum growth of the fungus coincidentally was obtained at pH 4.5 (1.04g/50 ml) when the maximum enzyme was achieved, followed by pH 4.0 (0.83 g/50 ml) as shown in Fig. 10. The alkaline pH 8.0 showed the least biomass production (0.33 g/50 ml). The difference in redox potential between the phenolic substrate and the T1 Cu could increase oxidation of the substrate at high pH values, while the OH⁻ binds to the T2/T3 tri-nuclear Cu centre. These opposing effects can assist in determining the optimal pH for laccase secretion [8].

3.7 Time Course Study of Laccase Production Using the Various Organic and Inorganic Feed Substrates

Laccase production has been reported to be influenced by culture conditions such as initial pH, metal ion, inducer compound, the carbon and nitrogen sources [80]. Time course study of laccase production by *P. ostreatus* was conducted before and after the medium optimization under static submerged fermentation. Thus, the culture conditions for the production of laccase using optimized physical and chemical parameters were investigated. The result of the time course study for laccase production using optimized medium parameters by the fungus is presented in Fig. 11. The appearance of laccase activity in the culture broth became significant after 24 h of cultivation and continued to increase with increase in cultivation time. The production of laccase peaked at the late stage of cultivation and this observation shows that the secreted enzyme was a secondary metabolite instead of primary metabolite. Laccase production was remarkably low in the medium for the first 3 days (1.23 U/ml) after which a steady increase was observed. Laccase activity in the optimized medium increased 3.0 fold compared to that of unoptimized medium (2.5 U/ml) with maximum laccase activity of 7.21 U/ml and 1.77 g/50 ml of fungal biomass on the 7th day of incubation. However, the laccase activity decreased after further cultivation time. This suggests an alteration to secondary metabolism and agreed with reports by Galhaup et al. [34], Patel et al. [3] and Liu et al. [25] on *P. ostreatus* and different fungi, respectively. The finding of this study differed with other previous reports on other fungi such as *Phlebia floridensis*, 20th day [81], *Pleurotus fossulatus*, 15th day [19] and *Monotospora* sp., 8th day [39]. Cilerdzic et al. [21] obtained maximum laccase production by *Ganoderma lucidum* on the 14th day of cultivation under submerged condition. Shankar and Shikha [82] reported 2.49-fold higher laccase production under optimized condition by *Peniophora* sp. Laccase production by *Corioloopsis gallica* under solid state fermentation was enhanced 3.2 times using RSM optimization [18]. Patel and Gupte [36] also reported 3.02-fold higher laccase production using optimized medium by *Tricholoma giganteum*. The maximum laccase activity obtained in the present study is higher than the maximum laccase activity of 4.96 U/ml for *Thermobifida fusca* using bagasse as carbon source [9].

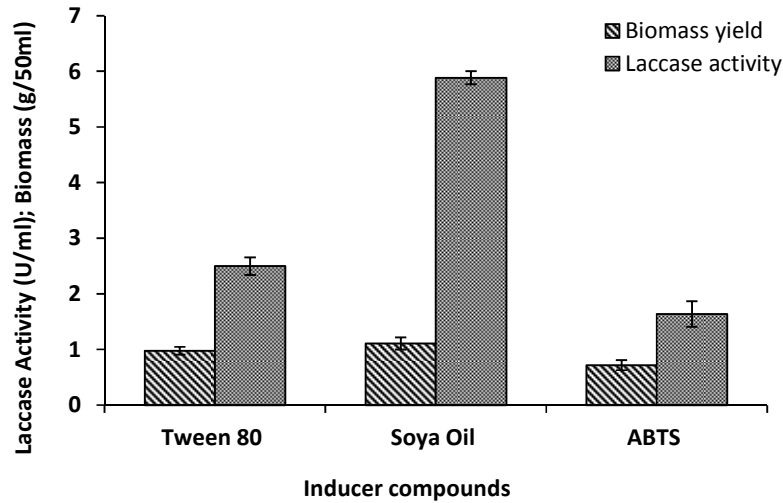


Fig. 8. Effect of Cu ion concentrations (0.01-0.1%w/v) on laccase production after 96 h cultivation

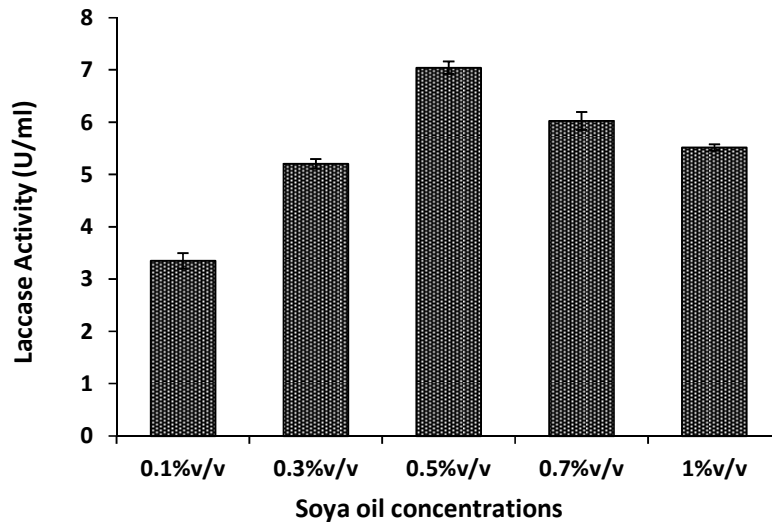


Fig. 9. Effect of soya oil concentrations (0.1-1.0%v/v) on laccase production after 96 h cultivation

Fungal biomass increased remarkably after 72 h incubation (1.34 g/50 ml), coinciding with continuous decrease in medium pH (4.2), a probable indication of the formation of organic acids by primary metabolism of *P. ostreatus*. This low pH encouraged maximum biomass (2.38 g/50 ml) before a steady pH increase from the 5th day (4.8) to the 9th day (8.6). This result indicated that maximum production of enzyme and biomass was obtained on the 7th day and 5th day, respectively. Patel and Gupte [36] reported that maximum production of laccase and biomass were both obtained on the 16th day of fermentation. Increase in fungal laccase and biomass yield was observed from pH 4.0 to 6.9,

but was reduced with further increase in pH. The maximum laccase activity of 7.21 U/ml and mycelial biomass of 1.77 g/50 ml was observed at pH 6.9. Such a phenomenon may be explained by the probable turn off of a large number of genes and a change in enzyme-related synthesis in the presence of increasing pH values [83]. Previous studies have suggested that the expression of laccases by most white rot fungi required an acidic initial pH, N-limitation and higher C/N ratio in cultures [84,85]. Wang et al. [39] reported that enzyme production was achieved with *P. fossulatus* in N-rich and C-limitation cultures at an alkaline pH.

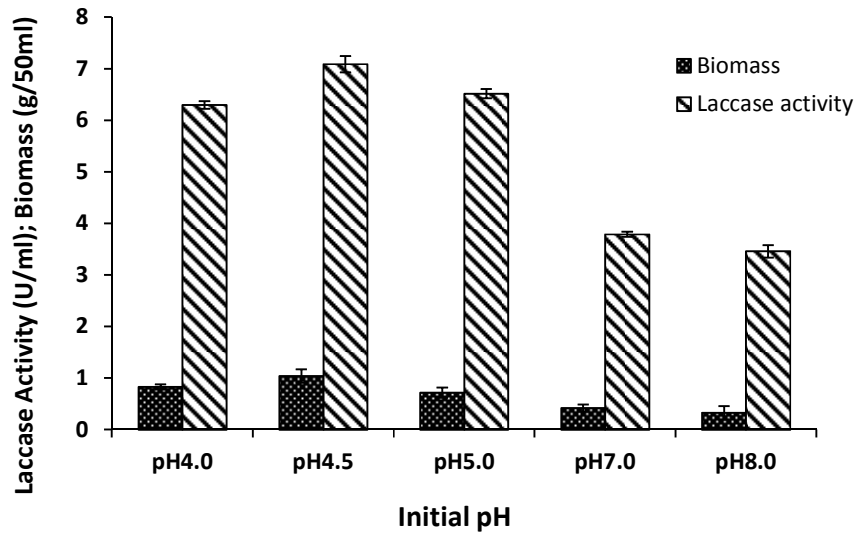


Fig. 10. Effect of different initial pH on laccase production and biomass yield after 96 h cultivation

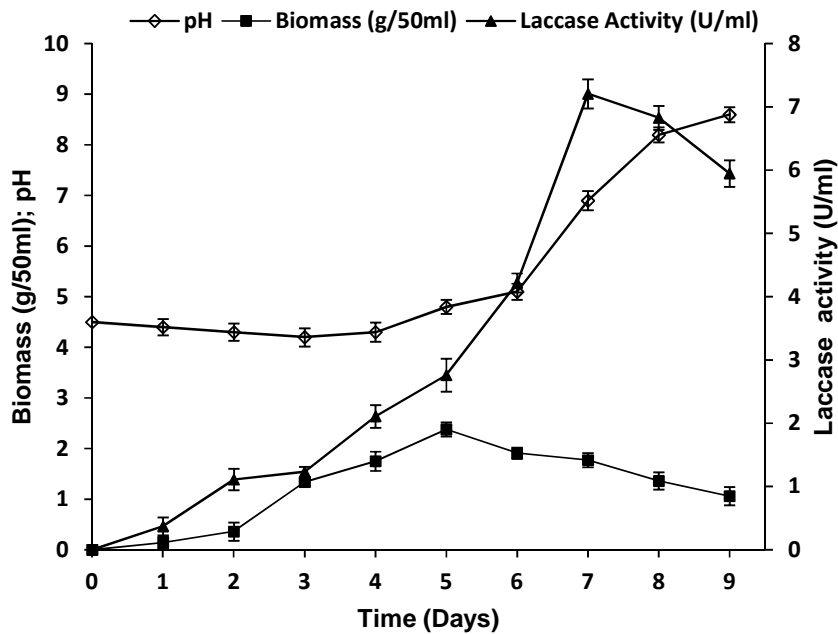


Fig. 11. A 9-day time course study of laccase production with optimized medium by *P. ostreatus*

4. CONCLUSION

This study revealed that interesting novel laccase producers can be still discovered from our local environments by very simple plate test methods, and also demonstrated the convenience of using Malt Extract Agar (MEA) as a screening medium.

The current study has shown the potential of *P. ostreatus* to produce laccase under static submerged fermentation. In addition, this study confirms and assesses the application of lignocellulosic substrates as cost effective and easily available solid substrates. The optimal conditions obtained were pH 4.5, soya oil 0.5%,

CuSO₄ 0.5%, NH₄Cl 0.1% and ground orange peelings 0.1%. However, the overall optimization of all the culture conditions increased laccase activity (7.21 U/ml) by 3-fold compared to unoptimized basal medium (2.5 U/ml) under static SmF using orange peelings as a lignocellulosic substrate. To the best of our knowledge, this is the first report on laccase production by this fungus using orange peelings under static SmF. The results obtained in this present study reveals that the laccase-producing potential of the local fungal strain *P. ostreatus* can be enhanced by optimizing the cultural conditions with safe and cost effective substrates. This study therefore, has found its relevance not only in the re-utilization of such agro industrial wastes (lignocellulosic substrates) for the production of laccase but also in alleviating pollution problems caused by their improper disposal. Moreso, this fungus can be further explored as a prospective candidate for the large scale laccase production for its various bioprocessing and industrial applications. Attempts to conduct molecular identification of the fungus and construct phylogenetic tree as well as efforts to purify and characterize the enzyme are currently on-going in our laboratory.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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