International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (1): 427-436
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Production of Laccase from Fungus *Pleurotus florida* Cultured in Different Agro-Wastes

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Received: 8 Oct 2018/ Accepted: 6 Nov 2018/ Published online: 01Jan 2019
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Abstract

The effluent of agriculture based industries is recyclable and could be converted to useful output. The main components of agro-wastes are lignin and cellulose which are not easily degraded. Microorganisms like bacteria and fungi secrete the enzymes which are able to oxidize such components. In the present study, optimization of the growth condition for the production of lignin degrading enzyme laccase by fungus *Pleurotus florida* using agrowaste such as baggase, orange peel, sweet lime peel, banana peel and grape stalk as substrates and its partial purification was undertaken. The fungus *P. florida* produces highest laccase activity (0.42 U/ml/min) using sweet lime peel than other substrates. Solid state fermentation for laccase production was optimised at 96 hrs fermentation time, 5 ml of inoculum volume and 60 % moisture content. The enzyme laccase produced by *P. florida* using sweet lime peel was investigated for the effect of nitrogen sources and inducers. Among different nitrogen sources tested, soy peptone showed highest laccase activity, similarly guaiacol increased laccase activity up to 0.48 U/ml/min. The enzyme laccase was partially purified with 60% ammonium sulphate and showed single subunit when it was subjected to SDS-PAGE with molecular a weight of 66kDa.

Keywords

Agrowastes, Laccase, *Pleurotus florida*, Solid state fermentation

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INTRODUCTION

The increased population, urbanization and modern industrialization result in huge amount of pollutants in the environment which is already considered as a major threat to the entire world. This leads to an increase in the attention for the development of various innovative, efficient and eco-friendly technologies to combat pollution. Agro-wastes are one of the major problems in agriculture industries. Recycling the agro-wastes could be useful output to a certain extent such as feed for animals and other

related products [1]. The main composition of agriculture waste was lignin and cellulose which are not readily degraded but microorganism utilizes such components as the energy sources by breakdown them with help of enzymes.

Recently different types of microorganism are being exploited for the production of various unusual enzymes which are capable of performing the difficult task under extreme conditions. Searching such kind of promising microbes is under limelight nowadays due to their ability to synthesize potential secondary metabolites [2, 3] and industrial enzymes [4] is under the limelight. This property of microbes has been in the limelight of the recent biotechnology innovations [5, 6]. The enzyme Laccases are multi-copper oxidases which were distributed broadly in plants, fungi and bacteria [7, 8]. Most of the laccases are blue colored which plays a major role in the direct oxidation of a variety of phenolic compounds. Similarly few of the yellow laccases were able to oxidize both phenolic and non-phenolic components [9, 10].

These laccase enzymes belong to oxidoreductase, which has the ability to oxidize a wide range of substrates, which does not require hydrogen peroxide during the oxidation process, however they utilize molecular oxygen as an electron acceptor. Thus they are considered as an ideal green catalyst which has major applications in different biotechnological industries including pulp, paper, food, pharmaceutical and textile [11, 12]. Laccase from white-rot fungi could be used for lignin degradation, dye bleaching, wood fiber modification and soil remediation [13]. The high cost factor for the production of enzymes using microorganisms limits the advantages to industrial applications. Strain selection and medium formulation play a major role in improving the laccase production in the fermentation process. The solid state fermentation process offers a considerable advantage over other fermentation processes such as high titer products, low water consumption, reduced energy requirements, simple media components [14].

The genus *Pleurotus* also commonly called as Oyster mushroom normally grows on wood. It includes a variety of species such as, *P. ostreatus, P. sapidus, P. cornucopiae, P. Platypus, P. sajor-caju, P. florida, and P. ostreatoroseus*. The fungus presents all around the world commonly found in forest regions. The organism produces several enzymes, importantly, Mn-peroxidase and laccase which involves the degradation of lignocellulosic components which enables them to grow in different agricultural residues with good adaptability under vaired climatic conditions [15]. A variety of substrates such as, paddy straw, wheat straw, sawdust, corn etc., can be utilized for the cultivation of the fungus [16]. These mushrooms were considered as rich in proteins, carbohydrates, fibres, vitamins and minerals and also have the pleasant taste with a high content of therapeutic properties [17, 18].

In this present study, we have investigated the production of laccase enzyme by *Pleurotus florida* using various agro wastes by solid state fermentation process. The cultural conditions for the production of laccase were studied and further, it was characterized.

MATERIALS AND METHODS

Fungus collection

The fungus *Pleurotus florida* was obtained from National Centre for Microbial Resources (NCMR), Pune, India. The fungal strain was sporulated in the freshly prepared potato dextrose agar (PDA) composed of (g L^{-1}): potato infusion, 200; dextrose 20 and agar, 15.0 (pH 5.6) at 25 ± 0.5 °C. The fungal strains were maintained by frequent sub culturing on PDA plates at 25 ± 1 °C and stored at 4 ± 1 °C till further use.

Preparation of substrates

Different agro wastes were pre-treated prior to the addition of production medium for the synthesis of laccase enzyme. The agro wastes baggase, orange peel (*Citrus sinensis*), sweet lime peel (*Citrus lemettioides*), banana peel (Musa Sp.) and grape stalk (*Nitis vinifera*) were procured from the local market, Chennai. The substrates, orange peel, sweet lime peel, and banana peel were subject to alkali treatment by soaking 10 g of each with 30 ml of 1% potassium hydroxide for one hour in order to neutralize organic acids. The substrates were then washed with distilled water, dried at room temperature for another 2 days. The substrates were autoclaved for 121°C for 15 min and used for the production laccase [19]. The other substrate such as, baggase and grape stalk was used in the dry state for the production of laccase.

Solid state fermentation for laccase production

The solid state fermentation for laccase production was performed in 250-ml Erlenmeyer flasks containing 5g of each substrates moistened with mineral salt solution [composition (gL^{-1}): 5, $Ca(NO_3)_2 \cdot 4H_2O$; 0.5, KH_2PO_4; 1, $MgSO_4 \cdot 7H_2O$] in solid substrate to moisture ratio of 1:3 (Sharma et al. 2005). The flasks with the substrate along with mineral salt medium were cotton plugged and autoclaved for 20 min at 121°C. Then the flasks were inoculated with five days old fungal strain of *P.*

florida on potato dextrose agar under aseptic conditions. The flasks were then incubated at the static condition for 5 to 7 days at 25°C. At the end of incubation, 50 ml of acetate buffer (50 mM, pH 5.0) was suspended to each flask and kept in the rotary shaker incubator for one hour at 180 rpm. The filtrate was then collected by separating the solids substrates by centrifuging the contents at 10,000 rpm for 10 min at 4°C. The obtained filtrate was then analysed for extracellular laccase enzyme activity and total protein estimation [20].

Laccase assay

Laccase assay performed by the following method: 1ml of culture filtrate was added with 3 mL of 10mM guaiacol in 100 mM acetate buffer (pH 5.0) containing 10% (v/v) acetone. The mixture was incubated for 15 min and after the incubation period, the solution was read at 470 nm using spectrophotometer (Shimadzu UV-160A). Enzyme unit was defined as the amount of enzymes needs to oxidize 1µmol of guaiacol [21].

Optimization of laccase production with different substrates by *P. florida*

Various parameters such as incubation period, inoculum volume and moisture content were standardized for the growth of *P. florida* and also for the production of laccase enzyme using solid state fermentation process. Triplicates were maintained during the optimization studies and the standardized protocols were followed for further studies on laccase production.

Effect of different nitrogen sources and inducer on the production of laccase by *P. florida*

Among the different agro wastes, the sweet lime peel showed better production of laccase enzyme and hence it was utilized for further laccase production studies. Different nitrogen sources such as peptone, soy peptone, beef extract, meat extract and malt extract (5 g/L) were used alternative to yeast extract to choose the good nitrogen source for laccase production in the sweet lime peel amended medium. Inducing agents such as vanillic acid, gallic acid, 1-hydoxybenzotriazole, guaiacol and *p*-anisidine were also studied by amending after 5 days of incubation period at the concentration of 1mM.

Protein estimation

Protein was determined using a micro-test based on the Bradford technique (BioRad) following the manufacturer's instructions; bovine serum albumin was used as the standard. The secreted protein concentrations during the fermentation process were determined and expressed in units of µg/mL [22].

Partial purification of laccase by ammonium precipitation method

Laccase was partially purified by the ammonium sulphate precipitation process. Different concentrations of ammonium sulphate (30%, 40%, 50%, 60%, 70% and 80%) were added to the 10 mL of crude extract. It was incubated for 12 hrs at room temperature. After incubation, the mixture was centrifuged at 12000 rpm for 10 minutes and the supernatant was collected, dialysed against acetate buffer (100mM, pH 5.0). The assay was also performed with filtrate by dissolving it in 0.1M sodium acetate buffer (pH 4.8) and the activities were recorded.

pH and thermal stability of isolated laccase

Partially purified laccase from *P. florida* was observed for pH sensitivity, thermal stability, substrate affinity and kinetics. Thermal tolerance of laccase assayed by holding the enzyme at different temperature ranging from 20 °C to 80 °C with the difference of 10 °C for 30 minutes. After incubation, the laccase assay was performed against the substrate.

The pH stability of laccase was tested dialysed sample incubated with different pH buffers such as pH 3-6.0 (1M sodium acetate buffer) and pH 7- 8 (phosphate buffer). Dialysed samples were re-equilibrate with acetate buffer and the laccase activity was observed

Analysis of partially purified laccase in SDS-PAGE:

Partially purified laccase was analyzed in SDS-PAGE system [23]. Partially purified laccase was mixed with sample buffer containing 5% of 2-mercaptoethanol, and it was heated for 1 minute at 100°C. Dis-continuous polyacrylamide gel was prepared using 5% stacking gel (pH 6.8) and a 10% separating gel (pH 8.8) in Tris-glycine buffer (pH 8.3). Heated laccase was loaded in wells of acrylamide gel and immediately electrophoresed at a constant voltage of 50V. The electrophoresed fractions were stained with coomassie brilliant blue (CBB) and the molecular weight of the unknown polypeptide bands in the gel was determined using standard SDS protein molecular weight markers.

RESULTS AND DISCUSSION

In the present study, different kinds of agro-wastes were used as substrates for laccase production by *P. florida.* Generally, the lignin and cellulose content agro wastes were found to be good substrates for the laccase production [24, 25, 26]. Among the various processes, solid state fermentation was considered as an effective and suitable for fungus production due to their natural condition for the growth and

development [27, 28]. In the present study, *P. florida* inoculated with agro wastes such as baggage, orange peel, sweet lime peel, banana peel and grape stalk with a suitable medium and various physical parameter were investigated. The laccase activity was observed and tested every alternative day.

Solid state fermentation with different substrates by *P. florida*

Among the five different agro-wastes used, the sweet lime peel shows highest activity (0.42 U/ml/min) of the laccase than any other substrate. It is followed by the orange peel with medium laccase activity of 0.34 U/ml/min. Other agro-wastes such as banana peel, baggage and grape stalk shows lower laccase activity of 0.29 U/ml/min, 0.28U/ml/min and 0.19U/ml/min respectively (Fig. 1). Previously, several researchers have investigated the utilization of agriculture wastes such as huge carbon, nitrogen content sources like rice husk, wheat husk, saw dust etc., in the microbial fermentation process [29]. The carbon repression effect enhances the production of laccase from lignocellulose wastes [30]. The enzyme laccase produced by white rot fungi requires the medium composed of carbon and nitrogen sources [31, 32]. Presence of carbon sources like lignin in the sweet lime peel and orange peel might be a reason for the high production of laccase activity than other substrates used in this study.

Optimization of laccase production in different substrate medium

The fermentation parameters such as fermentation time, inoculum size and moisture content of the medium were optimized for enhanced production of laccase from different substrates using *P. florida.* Production of enzymes from fungus takes long hours to produce; it depends on the metabolic activity of fungus. In the present study, each substrate produced an increased quantity of laccase at a different time of incubation period (Fig. 2). up to 48 hrs of fermentation time, there was no significant production of laccase in the medium by *P. florida.* After 72 hrs, the laccase activity was increased significantly in all substrate amended medium. Among the different substrates, the sweet lime peel shows the laccase activity of 0.36 U/min/ml. The grape stalk shows minimum laccase activity of 0.21 after 72 hrs. Similarly, at 96th hrs the laccase activity was increased in the substrate amended media such as sweet lime peel (0.46 U/min/ml), orange peel (0.34 U/min/ml) and banana peel (0.3 U/min/ml). Similar fermentation time period was reported by various researchers who have utilized agro wastes and highest laccase activity at 96 hrs of incubation period [5, 33]. After 120 hrs the laccase activity was reduced in the substrate medium of sweet lime peel, orange peel and banana peel. But interestingly, the enzyme activity was increased in substrate medium of baggage and grape stalk. The present study also reveals that depending on the substrate and fungus metabolic rate, the fermentation process gets varied which was well reported for different fungi [34, 35, 36, 37].

The inoculum size was optimized for all the tested substrate in the production medium. Different volumes of inoculum containing 10^6 to 10^8 spores/ml were investigated. The minimum volume of inoculum (3ml & 4ml) was not sufficient to produce the laccase activity at the end of the fermentation period. Maximum enzyme activity was observed for sweet lime peel, orange peel, banana peel, baggage and grape stalk (0.46 U/ml/min, 0.36 U/ml/min, 0.3 U/ml/min, 0.26 U/ml/min, 0.28 U/ml/min respectively) when the inoculum size was maintained at 5ml. Increasing the inoculum volume from 5 ml to 7ml decreased the activity of laccase in the production medium which may be due to insufficient substrate level to be utilized by fungus. Figure 3 reported that laccase activity production was found to be better when the inoculum size was maintained at 5 ml [26]. Fungal population competes for nutrients from the substrate when inoculum size is large in volume [36, 38].

Moisture content in fermentation medium was considered as an important parameter for the production of the enzyme. In the present work, different percentages of moisture content were tested for the enhanced laccase activity. Moisture content of 60% the substrates sweet lime peel (0.46 U/ml/min) orange peel (0.35 U/ml/min) banana peel (0.33 U/ml/min), baggage (0.28 U/ml/min) and grape stalk (0.3 U/ml/min) showed highest activity than other moisture contents (Fig. 4). Major fungal species produce laccase activity when the moisture content was at 60%-70% [38, 39]. *Neurospora sitophila* produce the highest laccase activity at 60% moisture content in different agro-waste substrates [26]. The fungus requires optimal moisture content for their effective growth. Different species survive at different moisture content ranging from 60% to 80% [38, 39, 40, 41]. Strong (2011) and Saffain et al. (2010) reported that 70% of moisture content yielded better laccase activity in their studies [39, 42].

Effect of different nitrogen sources and inducer on the production of laccase by *P. florida*

Among the different substrate tested, the sweet lime peel was chosen as a better substrate for laccase production and characterization studies. Nitrogen

source is very important for the enzyme synthesis using fungus. Here, different types of nitrogen sources were studied for better laccase activity in solid state fermentation condition. The different nitrogen sources tested were peptone, soy peptone, beef extract, malt extract and meat extract (5g/L), used as an alternative to yeast extract in the production medium. The results revealed that, the meat extract shows minimum laccase activity (0.34 U/ml/min), with soy peptone and malt extract fermented product shows the highest activity of laccases of 0.48 U/ml/min and 0.46 U/ml/min, respectively. Similarly, peptone and beef extract supplemented medium showed a laccase activity of 0.36 U/ml/min and 0.38 U/ml/min, respectively (Fig. 5). Malt extract can act as a nitrogen source yielded better production of laccase [24]. Similar studies also indicated that the malt extract act as a good enhancer for the production of laccase in the medium [43].

In general, fungal species produce a low level of extracellular laccase enzyme which could be enhanced by the addition of inducing agents in order to achieve maximum production of laccase [24, 30, 44]. Inducing agents such as vanillic acid, gallic acid, 1-hydoxybenzotriazole, guaiacol and p-anisidine were used to induce the laccase production by *P. florida.* Among these inducers gallic acid and guaiacol increase the production of laccase activity up to 0.46 U/ml/min and 0.48 U/ml/min, respectively than any other inducers tested (Fig. 6).

Partial purification of laccase produced by *P. florida*

Using sweet lime peel as a substrate in the production medium, the enzyme laccase was produced by the fungi *P. florida.* The crude extract was precipitated using a different concentration of ammonium sulphate. The 60% of ammonium provides the highest activity of laccase (3.65 U/ml/min) and the total activity decreased and the laccase specific activity was found to be increased (Table 1). Similar results were obtained when laccase was extracted from *Neurospora sitophila* [26].

Table 1: Summary of partial purification of laccase by *P. florida* using sweet lime peel

Fungal Substrate	Method	Vol. (mL)	Protein conc. (mg/mL)	Total activity	Specific activity (U/mg)	Purification folds
Sweet lime peel	Crude extract	250	10.32	631	0.52	1
	Ammonium precipitated (60%)	10	2.68	86.2	3.65	6.8

pH and thermal stability of isolated laccase

The thermal stability of isolated laccase enzyme was also studied. Laccase extracted from *P. florida* shows the highest activity when incubated at 30°C (Fig. 7) and also found to be stable. Increasing the temperature more than 30°C resulted in the decreased enzyme activity. Most of the fungus mediated laccase production at 30°C as an optimum temperature [45]. The activity of enzymes was found to be stable with the different organism at different temperatures. Thermo-stable laccase also reported from the fungi which were able to tolerate temperature even upto 60°C [46, 47].

The Laccase enzyme extracted using the sweet lime peel fermentation was analyzed for their pH and thermal stability. Different range of pH was tested for the laccase stability and a pH of 5 shows maximum activity with good stability (Fig. 8). The laccase activity was either reduced or not observed when the pH was altered from 5. The pH was found to be the most important factor whenever the activity of an enzyme is considered. Varying the pH may affect the stability of the enzyme which also depends on the enzyme produced by different fungi [48, 49, 50].

SDS-PAGE analysis of isolated laccase

The partially purified laccase was further subjected to SDS-PAGE analysis. The partially purified enzyme was estimated and 6µg was loaded on the polyacrylamide gel. At the end of electrophoresis the gel was stained with CBB. The results revealed that only one subunit in the laccase enzyme was able to observe in the gel and the molecular weight was estimated as 66kDa using standard molecular markers (Fig. 9).

Figure 1: Laccase production by *P. florida* using different agro-wastes as substrates

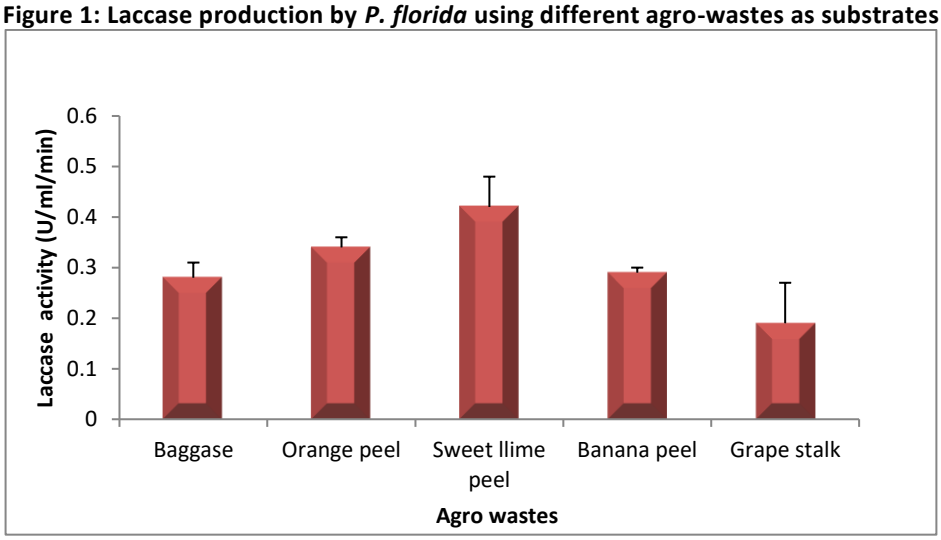


Figure 2: Effect of fermentation period on laccase production with different substrates

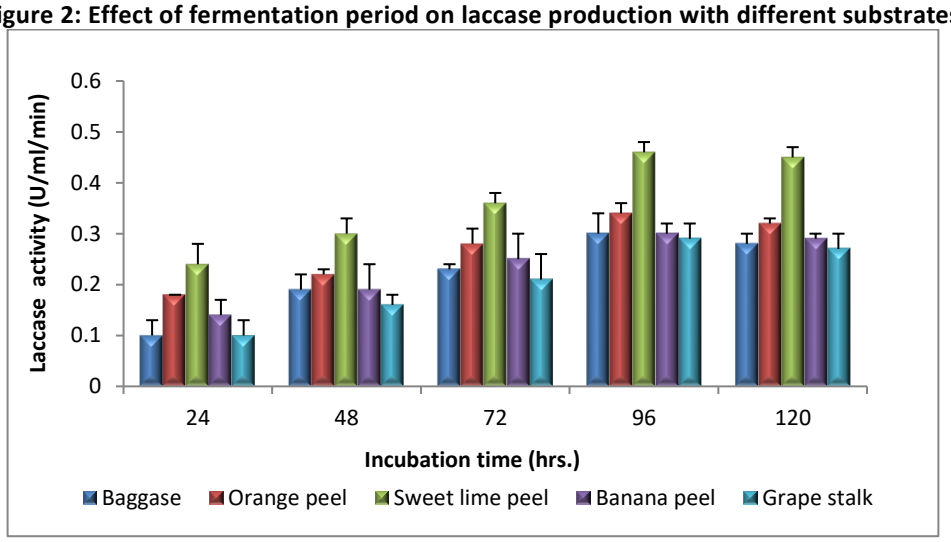


Figure 3: Optimization of inoculum size on laccase production

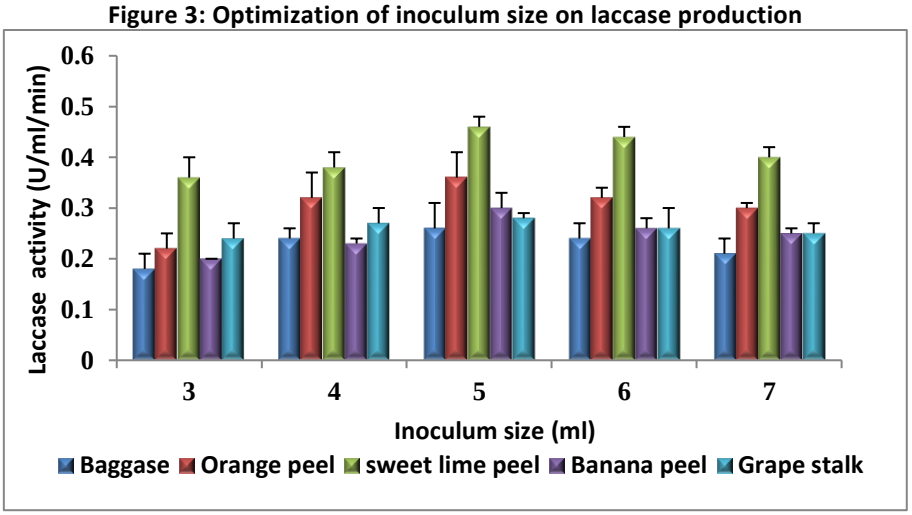


Figure 4: Effect of moisture content on laccase production

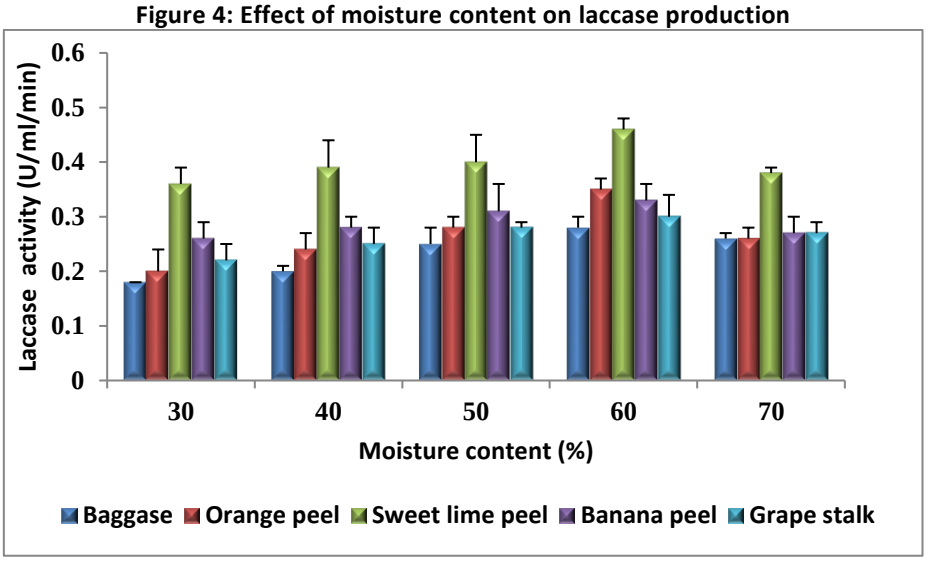


Figure 5: Effect of different nitrogen sources on laccase production

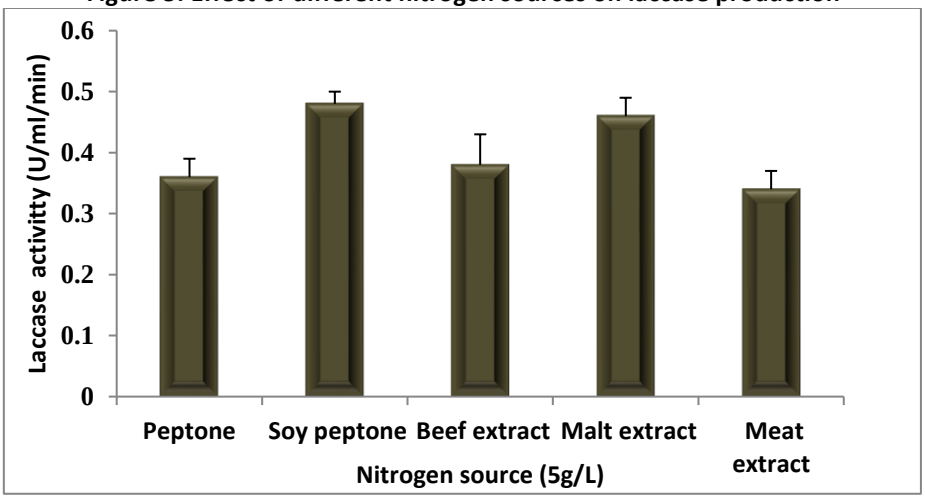


Figure 6: Effect of inducers on laccase production

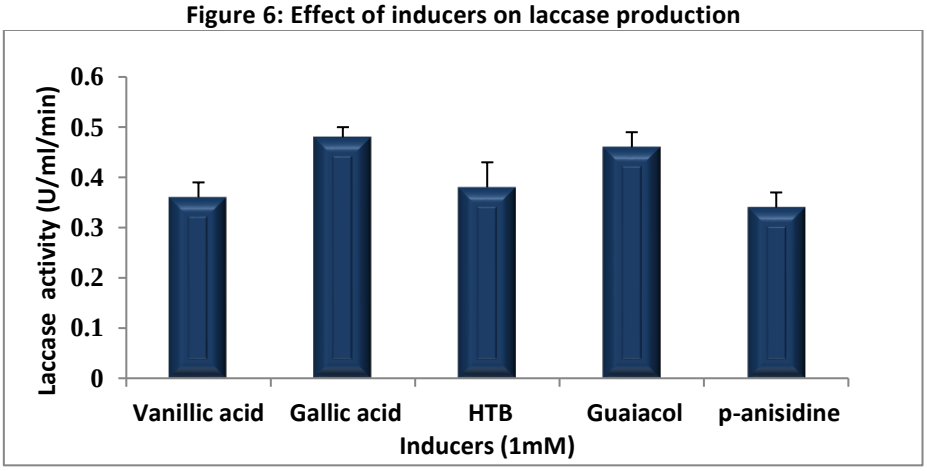


Figure 7: Thermal stability of laccase produced by *P. florida*

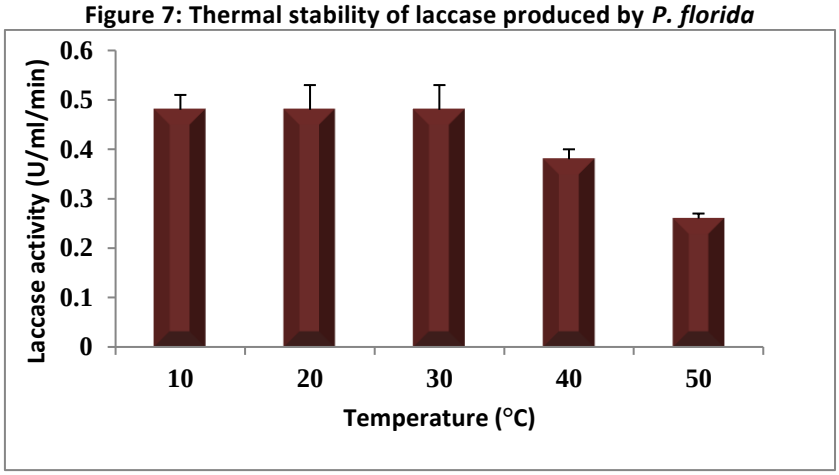


Figure 8: Effect of pH on laccase produced by *P. florida*

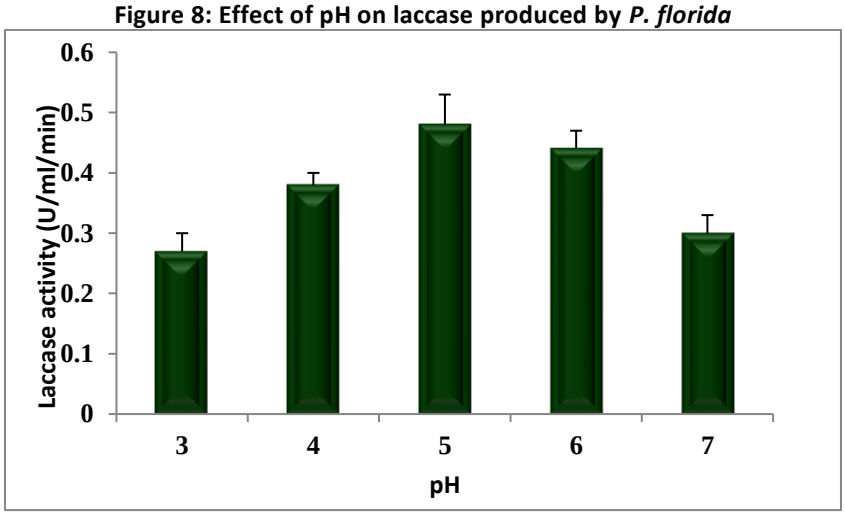
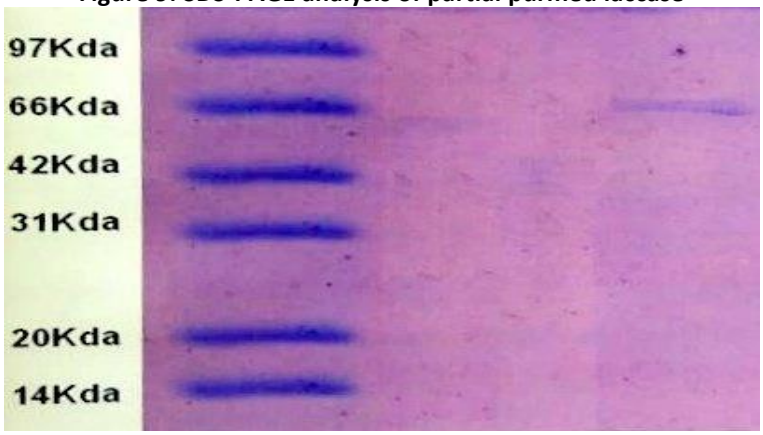


Figure 9: SDS-PAGE analysis of partial purified laccase



CONCLUSION:

Being Laccase used for various purposes in industries such as delignifiying, decolorizing and detoxifying agent there is need to study for higher yield and better activity. In the present study, the yield of lignolytic enzyme laccase by the fungus *P. florida* using different agro-wastes as substrates varied for each substrate were investigated. The production of laccase was affected by various parameters. Optimum fermentation time with required moisture

content produces maximum laccase production in solid state fermentation process. The sweet lime peel found to be a better substrate for higher laccase production in the present work which could be due to its huge content of lignin. Soy peptone and inducing agent such as guaiacol increase the laccase activity. The present study clearly demonstrated that the laccase production using agro-wastes in solid state fermentation yielded good productivity which could initiate the way for the utilization of organic wastes promoted enzyme production with cost-effective and eco-friendly approach.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest in the research.

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