



## PRODUCTION OF LACCASE FROM *GANODERMA* SP. BY USING AGRO-RESIDUAL WASTES

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### ABSTRACT

Solid-state fermentation (SSF) has emerged as a potential technology for the production of microbial products such as feed, fuel, food, industrial chemicals and pharmaceutical products. Its application in bioprocesses such as bioleaching, bio-beneficiation, bioremediation, biopulping, etc. has offered several advantages. Utilisation of agro-industrial residues as substrates in SSF processes provides an alternative avenue and value-addition to these otherwise under- or non-utilised residues. The aim of this study was to determine the most promising fungus, the best solid substrate and the optimal conditions for the production of laccase. A laccase producing fungus *Ganoderma* sp. was isolated and produces laccase by utilizing the agro-wastes. The study revealed that the rice bran was found to be the best supported lignocellulosic substrate for extracellular laccase production under SSF and optimum condition for highest laccase activity of pH and temperature were observed at 6.0 and 45°C respectively. The incubation periods for laccase maximum activity was at 8<sup>th</sup> day. Glucose and Peptone were the best supported carbon and nitrogen source for the maximum laccase activity.

**Keywords:** *Ganoderma* sp., Laccase, Solid State Fermentation.

### 1. INTRODUCTION

The enzyme production is a growing field of biotechnology. Annual world sales figures are close to billion dollars with increasing number of patents and research articles related to this field. Since the biotechnological applications require large amounts of low cost enzymes, one of the appropriate approaches for this purpose is to utilize the potential of lignocellulosic wastes, some of which may contain significant concentrations of soluble carbohydrates and inducers of enzyme synthesis ensuring efficient production of ligninolytic enzymes [1, 2]. Laccases have copper atoms at their catalytic sites and are oxidative enzymes (EC 1.10.3.2) which are widely found in many species of fungi, where they are involved in lignin degradation, in higher plants where they are involved in biosynthesis of lignin [3, 4], in bacteria [5]. Some species of fungi and insects produce laccases as intracellular proteins but most of the laccases are produced as extracellular proteins by all other types of producers [6]. The importance of laccase in various biotechnological areas underlines the need for expanding the spectrum of laccase-producing organisms and enhancing the potential of their laccase-

producing ability. The extracellular laccases obtained by basidiomycete fungi usually have low activities [7]. Their production, however, can be considerably stimulated in the presence of a wide variety of inducing substances. To enhance laccase production, various nutritional supplements, inducers such as veratryl alcohol, 2,5-xylidine, ferulic acid, guaiacol and lignin preparations (indulin, reax, lignosulphonate) have been used [8, 9].

In recent years, there has been an increasing trend towards efficient utilization of agro-industrial wastes for the production of value-added products such as edible mushrooms, ethanol, enzymes, organic acids etc. [10]. Solid state fermentation (SSF) is a technique in which fungi are grown on solid substrate or substrate moistened with a low quantity of mineral salt solution and it has a great potential to produce enzyme especially where the fermented raw materials are used as a source of nutrients for the fungi. The enzymes produced by this method have several applications in several fields including food and fermentation industry. These enzymes are also used to prepare several bioactive compounds. SSF system is much better than the submerged system because a number of reasons. The benefits of SSF over SMF include

the high production of the enzyme and fewer effluent generations. Moreover, comparably simple equipment is required for SSF [11]. The present work was undertaken to examine the effectiveness of selected agro-wastes in production medium and optimizing the parameters using agro-wastes for maximizing laccase production by *Ganoderma* sp.

## 2. MATERIAL AND METHODS

### 2.1. Microorganism

Selected organism was screened for laccase production on potato dextrose agar plates (PDA) containing indicators namely, guaiacol, ABTS, syringaldazine and tannic acid. Isolated organism showed positive reaction for ligninolytic enzymes (Lac, MnP and Lip) was maintained on PDA plates at 30°C and stored at 4°C and identified as *Ganoderma* sp. [12, 13].

### 2.2. Screening of different lignocellulosic substrates for extracellular laccase production

The mentioned agro-residual wastes were used for the initial screening: rice bran, paddy straw, sugarcane bagasse and saw dust. All of them were locally procured and were sterilized at 121°C and 15lb pressure for 20 mins and were used for the study [14].

### 2.3. Media preparation

The media was prepared by adding 2% of each of the agro-wastes to the Mineral Salt (MS) medium [15]. The media was then sterilizing at 121°C and 15lb pressure for 20 minutes. This agro-wastes mineral salt (AWMS) media was used for the study. The 250 ml conical flasks with 100 ml of the above AWMS media and were inoculated with well grown fungal discs from PDA plates and the flasks were adjusted at a pH of 6 and incubated at 40°C for 5 days and the enzyme extraction and assay were done as guaiacol assay method. The best agro waste which supports the highest laccase assay were selected and used for the optimization study.

### 2.4. Laccase Harvesting

After some specified days of incubation, laccase was extracted by a simple contact method. For this purpose, 100ml of sodium acetate buffer (pH 5.5) was added in the flasks. The flasks were placed on incubator shaker at 150 rpm for 1 hour. Mixture was then filtered with filter paper and the filtrate was centrifuged at 10,000 rpm for 10 minutes at -10°C to remove all spores and other

impurities. The supernatant was collected and subjected to laccase assay [16].

### 2.5. Effect of initial moisture content on laccase production

Investigation of the influence of the initial total moisture content (before autoclaving) of the substrate was carried out under various initial moisture content adjusted with salt solution. Samples containing 5 moisture levels (30%, 45%, 50%, 65% and 70%) were prepared by moistening 5 g of studied substrates with salt solution [17]. The optimum initial moisture content of solid substrate achieved by this step was fixed in subsequent experiment. After soaking, the sample was again dried as described above and percent moisture content was calculated as follows, Percent of moisture content (initial) of solid medium =  $(\text{wt. of the rice bran} - \text{dry wt.}) \times 100 / \text{dry wt.}$  [18, 19].

### 2.6. Effect of physical factors on laccase production

The effect of pH on laccase production in rice bran broth was carried out by incubating with different initial pH. The experiments were carried out individually at various pH ranging from 4 to 9. The enzyme assay was carried out individually after 8 days of incubation.

The effect of various temperature ranges on laccase production in rice bran broth was studied by inoculating the *Ganoderma* sp. and the incubating the flasks at different temperatures viz., 30°C, 40°C, 45°C, 50°C and 55°C. The flasks were incubated for 8 days and assay was carried out.

### 2.7. Effect of incubation period for laccase production

The effect of incubation period on laccase production in rice bran broth was studied by inoculating with *Ganoderma* sp. and incubated at room temperature for various time intervals. The enzyme was extracted and its activity was determined.

### 2.8. Effect of carbon and nitrogen source for laccase production

The effect of different carbon and nitrogen sources on laccase production was studied. Different carbon sources namely glucose, mannose, cellobiose and maltose were tested for laccase production by the *Ganoderma* sp. Organic and inorganic nitrogen sources like ammonium nitrate, peptone, and urea were amended to the culture

medium with the *Ganoderma* sp. for laccase production. The flasks were incubated at 40°C.

### 2.9. Extracellular laccase assay

The Laccase activity was assayed at room temperature by using 10 mM Guaiacol in 100 mM sodium acetate buffer (pH 5.0). The reaction mixture contained 3.0 ml acetate buffer, 1.0 ml Guaiacol and 1.0 ml enzyme source. The change in the absorbance of the reaction mixture containing guaiacol was monitored at 470 nm for 10 mins of incubation using UV Spectrophotometer. Enzyme activity is measured in U/ml which is defined as the amount of enzyme catalysing the production of one micromole of coloured product per min per ml [20].

Calculation: Volume activity (U/ml) =  $(\Delta A_{470\text{nm}}/\text{min} \times 4.0 \times V_t \times \text{dilution factor}) / (\epsilon \times V_s)$

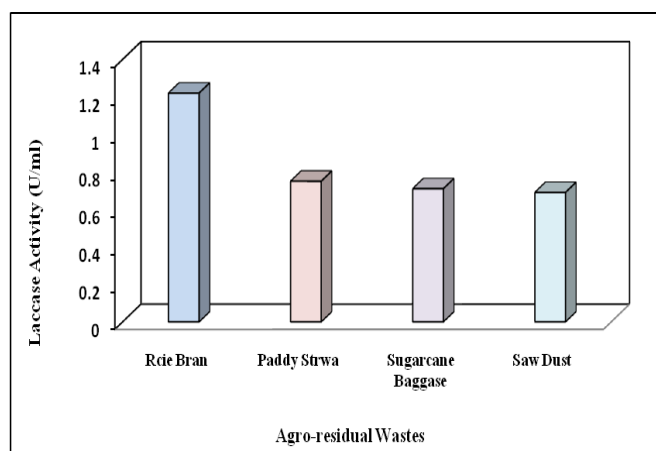
Where,  $V_t$  = final volume of reaction mixture (ml) = 5.0  $V_s$  = sample volume (ml) = 1.0  $\epsilon$  = extinction coefficient of guaiacol = 6,740/M/cm 4 = derived from unit definition & principle

## 3. RESULTS AND DISCUSSION

One of the effective approaches to reduce the cost of enzyme production was to replace pure carbohydrates as substrates with relatively cheaper materials namely lignocellulosics. Majority of organic materials available in nature like polysaccharides, proteins and lignin were polymeric in structure.

### 3.1. Selection of lignocellulosic substrates

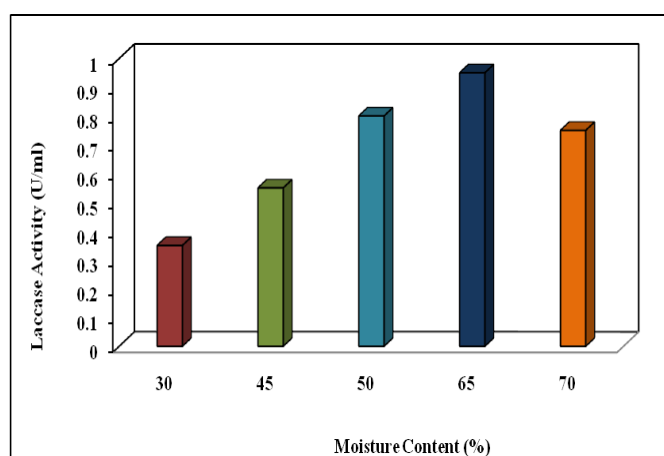
The choice of a suitable agro-residual waste for a fermentation process is an important factor for the microbial growth and enzymes secretion [21]. Agro-residual wastes like rice bran, rice straw, sugarcane bagasse and saw dust waste were screened for laccase production. In the present study, total 04 substrates as stated in the materials and methods, were tested for the suitability of maximum laccase production by *Ganoderma* sp. Among all studied substrates rice bran has yielded the higher amount of laccase at 5<sup>th</sup> day of incubation (Fig. 1). Paddy straw is also best substrate for laccase production next to the rice bran. As per industrial point of view incubation time is also very important, so less incubation and higher amount yielding rice bran was considered for the further studies. The high laccase yield reported on rubber tree sawdust by *P. sajor-caju* [22]. These results indicate that the production of lignolytic enzymes by SSF is dependent on the substrate material as well as the microbial strain employed for the production.



**Fig. 1: Screening of lignocellulosic substrates for laccase production by *Ganoderma* sp.**

### 3.2. Influence of initial moisture content on laccase production

In the present study, an initial moisture content of 65% was found optimum for laccase production (Fig. 2). The laccase production varied significantly between 65% and all other percentages of moisture content with an exception being at 70% of moisture content. When the moisture content less than 60% reduced laccase production was observed. This might be due to at lower moisture contents result in decreased solubility of nutrients, lower substrates swelling and higher water tension [23]. *P. ostreatus* HP-1 at 60% moisture content is suitable for higher amounts of laccase secretion during growth on wheat straw [24]. 66% IMC is best suitable for maximum laccase production by *P. ostreatus* under SSF conditions using wheat straw as a substrate [25].



**Fig. 2: Effect of initial moisture content for laccase production from *Ganoderma* sp.**

### 3.3. Optimum pH and temperature for laccase

In order to investigate the effect of the initial medium pH on laccase production by the *Ganoderma* sp., the production medium was adjusted to different pH values ranged between pH 4 and pH 8. The result showed that the maximum laccase production was obtained when the pH value of the production medium was adjusted to 6.0 (Fig. 3). Thurston, (1994) reports indicated that the initial pH between 4.5 and 6.0 was suitable for enzyme production [26]. The pH variation during fermentation depended highly on the nature of microorganism used. With *Aspergillus* sp., *Penicillium* sp., and *Rhizopus* sp. a rapid drop in pH below 3.0 was reported due to the possibility of secretion of organic acids. In the case of *Trichoderma* sp., *Sporotrichum* sp. and *Pleurotus* sp. the pH was more stable between 4 and 5 during fermentation [27].

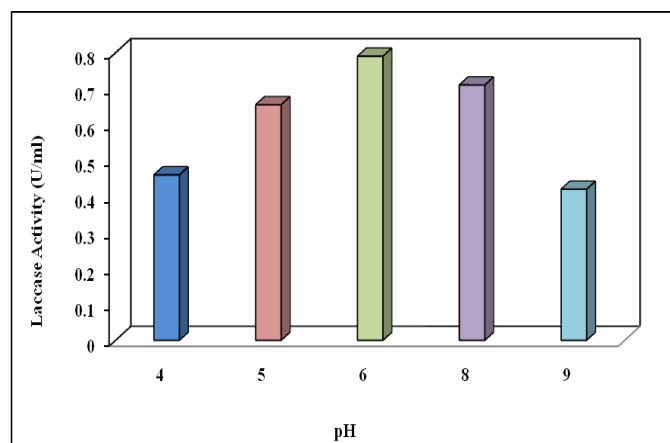


Fig. 3: Effect of pH on laccase production by *Ganoderma* sp.

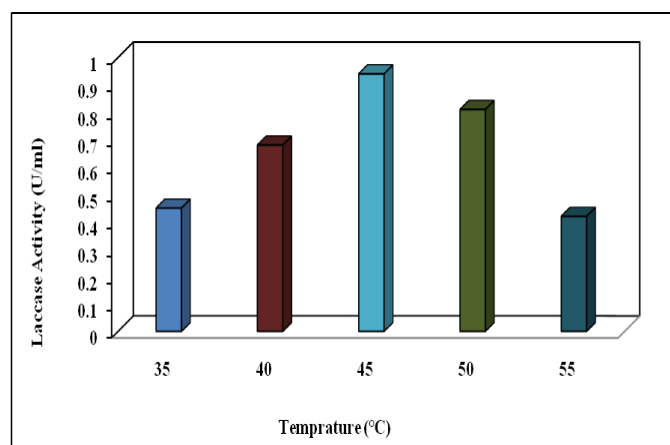


Fig. 4: Effect of temperatures on laccase production by *Ganoderma* sp.

Different incubation temperatures (35, 40, 45, 50 and 55 °C) were used to determine the optimum temperature for laccase production by *Ganoderma* sp. The result showed that laccase specific activity is increased with temperature at 45°C (Fig. 4) and decreased at higher temperatures. The laccase of *Trametes hirsuta* showed maximum activity at 40°C [28]. The optimum temperatures previously reported for other fungal laccases, ranging from 40°C to 50°C [29].

### 3.4. Effect of incubation period on laccase production

The present experiment aims to determine the optimal incubation period for laccase production by *Ganoderma* sp. The results indicate that the 8<sup>th</sup> day of incubation showed maximum laccase production (Fig. 5) and below or above this incubation value a considerable. After 10<sup>th</sup> days of incubation, the enzyme might be inactivated by the secretion of proteases by the fungus or due to the denaturation of the enzyme protein [30]. Chiranjeevi et al., (2014) have reported that the maximum level of laccase production was observed at 18<sup>th</sup> days of incubation period by *Pleurotus ostreatus* [31]. Nadeem and Sheikh, (2014) have reported that the maximum level of laccase production was observed at 16<sup>th</sup> days of incubation period by *Pleurotus ostreatus* [32].

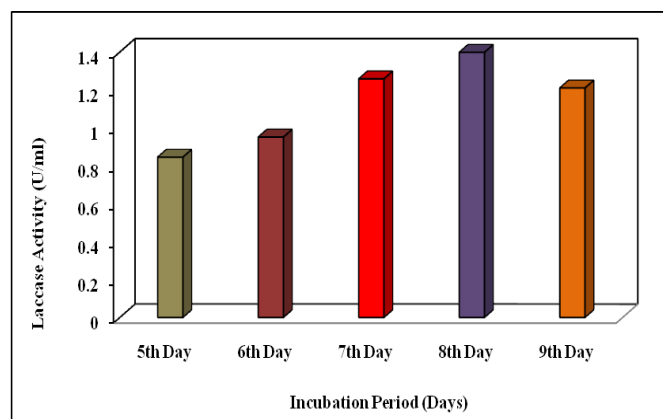


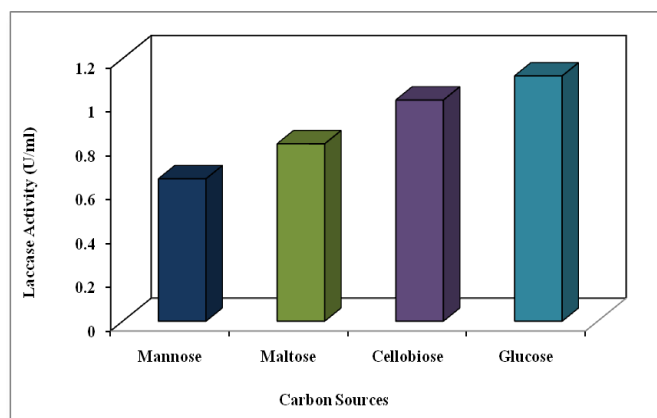
Fig. 5: Effect of incubation period on laccase production by *Ganoderma* sp.

### 3.5. Effect of carbon and nitrogen sources on laccase production

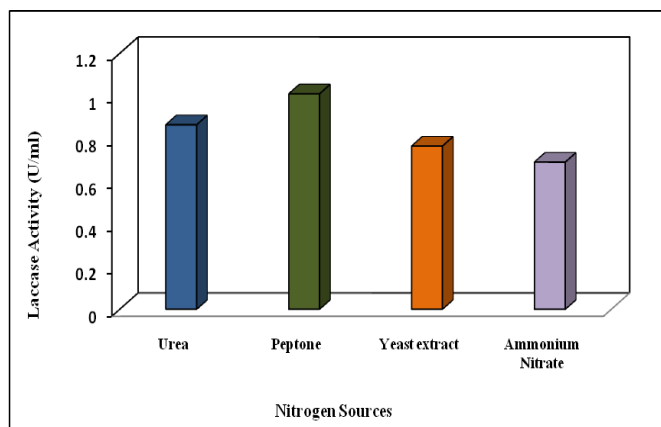
The carbon source is powerful nutrition regulation factors for producing the lignolytic enzymes. Glucose was the most enhancing carbon supplement; it produced maximum laccase production by *Ganoderma* sp. (Fig. 6). Chiranjeevi et al., (2014) have reported that the glucose

supported maximum of laccase production by *P. Ostreatus* [31]. Patel et al (2009) observed addition of glucose to the wheat straw raises the laccase production by *P. ostreatus* HP-1 [24].

The results showed that the peptone was supported the maximum laccase production (Fig. 7). These results are in accordance with the findings where the authors observed an enhanced laccase production was with peptone followed by urea supplementation [31].



**Fig. 6: Effect of carbon source on laccase production by *Ganoderma* sp.**



**Fig. 7: Effect of nitrogen source on laccase production by *Ganoderma* sp.**

#### 4. CONCLUSION

From the results obtained, it can be concluded that *Ganoderma* sp. is capable of utilizing the rice bran and produce the laccase under SSF conditions. In optimizing SSF process, the factors such as the carbon source and the levels of the nitrogen could influence the growth and production of the metabolites. It followed the trend of the rice bran which has more amount of total

carbohydrates and nitrogen content gave the more yield of laccase by the *Ganoderma* sp. After optimization of the fermentation conditions like pH, temperature, incubation period 100% improvement was noticed. These promising results suggest the application of the system to industrial-scale operation in order to produce laccase enzyme economically challenging.

#### Conflict of interest

None declared

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