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Research Article

PRODUCTION OF LACCASE BY *GANODERMA Sp.* IN SUBMERGED FERMENTATION

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ABSTRACT

The present study focuses on enhancing the process parameters to achieve the maximum production of extracellular laccase by Submerged Fermentation (SmF). Culture conditions and medium composition play a major role in enzyme expression. Laccases are generally produced during the secondary metabolism of white-rot fungi growing on natural substrate or in submerged culture. Physiological demands vary among white-rot fungi and considerable research has been done on the influence of agitation, pH, temperature, carbon, nitrogen sources, microelements and their levels. Submerged fermentation involves the nurturing of microorganisms in high oxygen concentrated liquid nutrient medium. The production parameters production medium, pH, temperature, solvents, carbon and nitrogen sources were studied. Yeast extract peptone dextrose-Copper sulphate (YPD-Cu) medium good supporter for production of laccase. pH and temperature for highest laccase activity was found to be 6.0 and 4°C respectively. Starch and peptone were the most suitable carbon and nitrogen sources for maximum laccase production. Ethanol was the most suitable solvent for laccase production.

Keywords: *Ganoderma* sp., Laccase, Submerged Fermentation, Physical and Nutritional Parameters.

1. INTRODUCTION

In the recent years, enzymes have gained great importance in Industries; laccases are one among them which are widely present in the nature. Laccases are the oldest and most studied enzymatic systems [1]. These enzymes contain 15-30% carbohydrate and have a molecule mass of 60-90 kDa. These are copper containing 1,4-benzenediol: oxygen oxidoreductases (EC 1.10.3.2) found in higher plants and microorganisms. These are glycosylated polyphenol oxidases that contain 4 copper ions per molecule that carry out 1 electron oxidation of phenolic and its related compound and reduce oxygen to water [2, 3]. When substrate is oxidized by a laccase, it loses a single electron and usually forms a free radical which may undergo further oxidation or non-enzymatic reactions including hydration, disproportionation, and polymerization [4]. These enzymes are polymeric and generally contain 1 each of type 1, type 2, and type 3 copper centre/subunit where the type 2 and type 3 are close together forming a trinuclear copper cluster.

Laccases are widely distributed in higher plants, bacteria, fungi, and insects. In plants, laccases are found in cabbages, turnip, potatoes, pears, apples, and other vegetables. They have been isolated from Ascomyceteous, Deuteromycteous and Basidiomycetous fungi to which more than 60 fungal strains belong [3]. The white-rot Basidiomycetes fungi efficiently degrade the lignin in comparison to Ascomycetes and Deuteromycetes which oxidize phenolic compounds to give phenoxy radicals and quinines [5]. Laccases are the enzymes which are secreted out in the medium extracellularly by several fungi [6] during the secondary metabolism but not all fungal species produce laccase such as Zygomycetes and Chytridiomycetes [7]. The production of laccase by soil as well as some freshwater Ascomycetes species [8].

Laccase has a wide range of applications it needs to be produced in higher quantity. Submerged fermentation involves the nurturing of microorganisms in high oxygen concentrated liquid nutrient medium. Viscosity of broth is the major problem associated with the fungal submerged fermentations. When fungal cell grows,

mycelium is formed which hinders impeller action, due to this limitation occurring in oxygen and mass transfer. For dealing with this problem, different strategies have been employed. Bioreactor operates in continuous manner for obtaining high efficiency. In this *Trametes versicolour* is employed which decolorizes the synthetic dye, and for this purpose pulsed system has been developed [9-11]. Broth viscosity, oxygen, and mass transfer problems are also solved by cell immobilization. Luke and Burton, (2001) reported that continuous laccase production takes place without enzyme deactivation for a period of 4 months due to the immobilization of the *Neurospora crassa* on membrane [12]. For bioremediation of pentachlorophenol (PCP) and 2,4-dichlorophenol (2,4 DCP), nylon mesh is used for comparing the free cell culture of *T. versicolour* with immobilized cultures. Couto *et al.*, (2004) and Sedarati *et al.*, (2003) investigated that, in fixed bed bioreactors, stainless steel showed the highest laccase activity among different synthetic materials which were used as carriers for the immobilization of *Trametes hirsute*. The most effective way of producing laccase is Fed-batch operation through which the highest laccase activity can be obtained [13, 14].

Hence, the present investigation on production of laccase by *Ganoderma* sp. and reported various parameters which affects the production of laccase in submerged fermentation.

2. MATERIALS AND METHODS

2.1. Microorganism

Pure culture of *Ganoderma* sp. was isolated and confirmed for laccase production and the strain was maintained on PDA plates at 30°C and stored at 4°C for further studies [15].

2.2. Extracellular laccase assay

The laccase activity was assayed at room temperature by using 10 mM Guaiacol in 100 mM sodium acetate buffer (pH 5). The reaction mixture contained 3 ml acetate buffer, 1 ml Guaiacol and 1 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^{-1} which is defined as the amount of enzyme catalyzing the production of one micromole of coloured product per min per ml [16, 17].

Calculation: Volume activity (U ml^{-1}) = $\frac{\Delta A470\ nm\ min^{-1} \times 4 \times Vt \times dilution\ factor}{€ \times Vs}$

Calculation

Where,

Vt = final volume of reaction mixture (ml) = 5.0

Vs = sample volume (ml) = 1

€ = extinction co-efficient of guaiacol = 6,740 $M^{-1}\ cm^{-1}$

4 = derived from unit definition & principle

2.3. Screening of different media on laccase production

Laccase production was studied in three different media. Two media used in the study were those of Yeast extract peptone dextrose-Copper sulphate (YPD-Cu) medium and Glucose Peptone Broth (GPB) medium and Potato Dextrose Broth (PDB) [18]. Yeast extract peptone dextrose-Copper sulphate (YPD-Cu) medium contained (in gl^{-1}) 20 glucose, 5 peptone, 2 yeast extract, 100 mg l^{-1} copper sulphate and Glucose Peptone Broth (GPB) medium contained (in g l^{-1}) 10 glucose, 3 peptone, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4, 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$, 0.01 $CuSO_4$.Potato Dextrose Broth medium contained (in g l^{-1}) Peeled Potato, Dextrose and all these media were adjusted to pH 6. Each medium (100 ml) was dispensed into 250 ml conical flask and autoclaved at 121°C for 15 mins. The fungus mycelium disc (6 mm) was inoculated into each of the conical flask and added 100 µl of guaiacol under sterile condition and they were then incubated on a rotary shaker at 120 rpm. After 5 days, 5 ml of the culture filtrate was taken and centrifuged at 10,000 rpm for 10 mins. The clear supernatant was used as a crude enzyme source for determining laccase activity.

2.4. Physical parameters effect on laccase production

2.4.1. Effect of pH and temperature on laccase production

The effect of pH was studied by adjusting the pH of the laccase production medium to different pHs ranging 3-8 inoculated with the mycelium and added 100 µl of guaiacol.

The effect of temperature was studied by adjusting the temperature of the laccase production medium to mycelium was inoculated in the production medium at different temperatures ranging from 30-70 °C.

2.5. Nutritional parameter effect on laccase production

2.5.1. Effect of carbon and nitrogen sources

Different carbon sources namely starch, sucrose, maltose, glucose and mannitolwere tested for laccase

production by the test strain. Organic and inorganic nitrogen sources like ammonium nitrate, ammonium chloride, peptone, urea and yeast extract were amended to the culture medium with the test strain. The flasks were incubated at 37°C for 5 days and added 100 µl of guaiacol. The enzyme assay was performed as Guaiacol assay method.

2.5.2. Effects of solvent systems

The effect of different solvents like ethanol, formic acid, isopropanol, methanol and tolueneon the production of laccase was studied. The sources were amended in the production medium. The mycelia of 5 day old culture was transferred to Erlenmeyer flasks (250ml) containing 100 ml of production medium. Flasks were incubated at 37 °C and analyzed for enzyme activity.

3. RESULTS AND DISCUSSION

3.1. Screening of different media on laccase production

High production of laccase was obtained with YPD-Cu medium followed by GPB medium and PDB medium (Fig. 1). In this study, the medium containing copper at lower concentration induced laccase production. Mansur *et al.,* (1998) reported that laccase production is induced by high medium nitrogen content detected in Basidiomycete I-62 CECT 20197 [19]. Periyasamy, (2012) have reported that optimum laccase production by Slomczynski *et al.,* (1995) by *Pleurotus* sp [20].

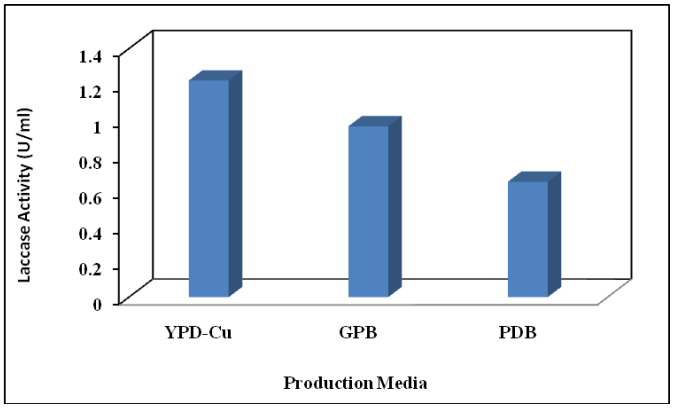

Fig. 1: Screening of different media for laccase production

3.2. Effect of different pH on laccase production

The *Ganoderma* sp. was able to show a maximum activity pH 6.0 (Fig. 2). Similarly, Sivakumar *et al.,* (2010) have reported that optimum laccase production at pH 6 by *Ganoderma* sp [21].The pH of the culture medium is critical and plays a significant role in the growth and laccase production of the organism. Fungi are grown in a medium with pH 5.0 laccase will be produced in excess [22]. Most reports indicated initial pH levels set between pH 4 and 6 prior to inoculation, but the levels were not controlled during most cultivations [23]. The optimum pH of laccase production, as reported in many fungi, falls between 5.0 and 6.0 [24, 25].

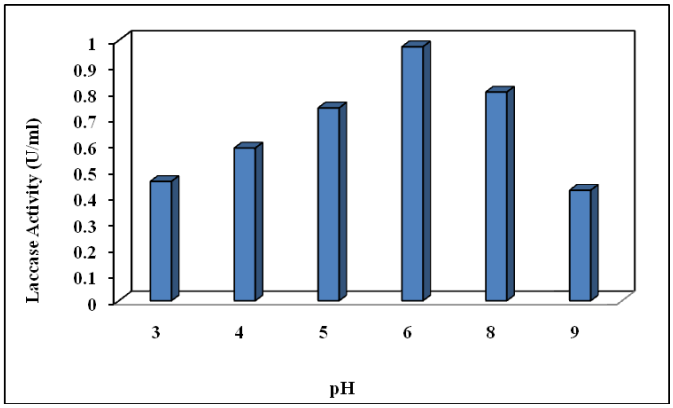

Fig. 2: Effect of pH on laccase production

The *Ganoderma* sp. was able to show a maximum activity at 40°C (Fig. 3). Periyasamy, (2012) have reported that optimum laccase production at 50°C by *Pleurotus* sp [20]. The effect of temperature is limited in case of laccase production. The optimal temperature of laccase differs greatly from one strain to another. The laccase from *P. ostreatus* is almost fully active in the temperature range of 40°C-60°C, with maximum activity at 50°C. The activity remains unaltered after prolonged incubation at 40°C formore than 4h [26]. Farnet *et al.,* (2000) found that preincubation of enzymes at 40°C and 50°C greatly increased laccase activity [27]. Nyanhongo *et al.,*(2002) showed that laccase produced by *T. modesta* was fully active at 50°C and was very stable at 40°C but half-life decreased to 120 min at higher temperature (60°C) [28].

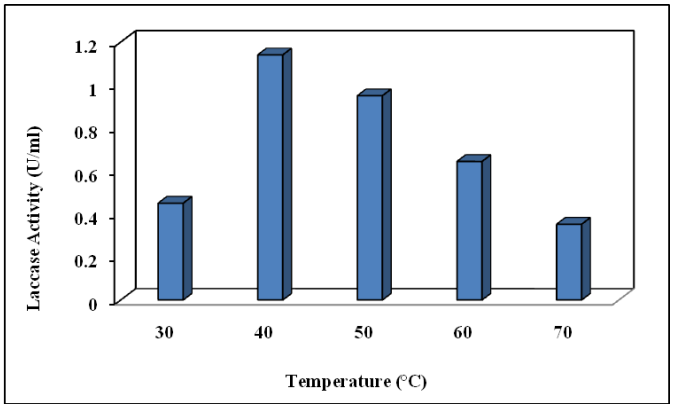

Fig. 3. Effect of temperature on laccase production

3.3. Effect of carbon and nitrogen sources on laccase production

The *Ganoderma* sp. was subjected to different carbon sources and starch which was recorded maximum laccase activity (Fig. 4). Sivakumar *et al.*, (2010) found starch as an effective carbon source for the production of laccase from *Ganoderma* sp [21]. The mannitol, cellobiose, and glucose ensured highest laccase activity of *P. ostreatus* IBB 191 [29]. Significant laccase formation by *Trametes pubescens* was shown in the presence of cellobiose and glucose, while poorly utilized lactose and a-cellulose resulted in low laccase levels [30]. Recently, Stajic′ *et al.,* (2006) observed the highest laccase activity in the presence of mannitol, glucose, and sodium gluconate in two strains of *P. ostreatus* [31].

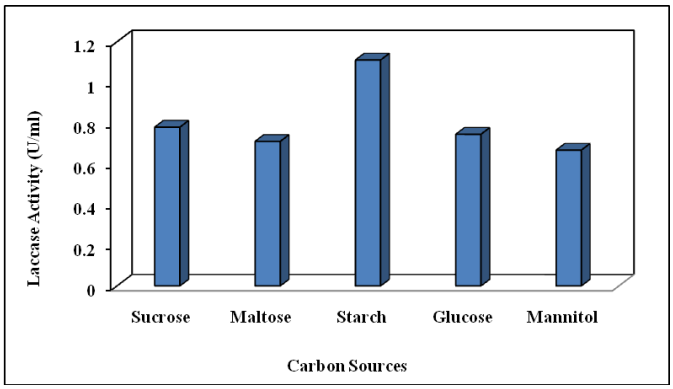

Fig. 4: Effect of carbon source on laccase production

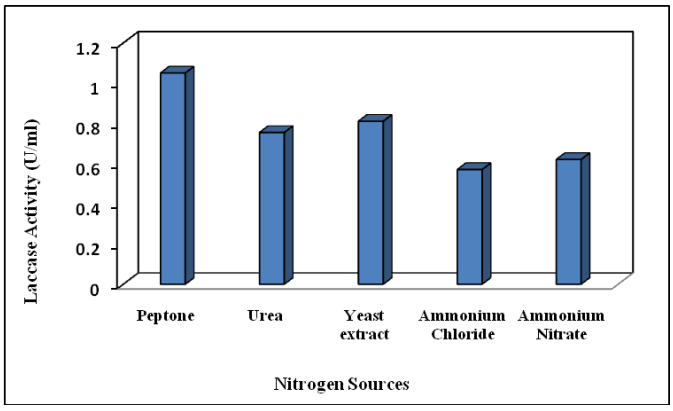

Fig. 5: Effect of nitrogen source on laccase production

Ganoderma sp. showed maximum laccase activity with peptone (Fig. 5). Similarly, Ruchika *et al.,* (2015) reported that the *Polyporus*sp. and *Phanerochaete* sp. showed maximum laccase activity with peptone and NH_4Cl respectively [32]. Periyasamy, (2012) reported that maximum laccase activity with peptone [20]. Nitrogen is a major element occurs in all living organisms. It plays a crucial role in the living organisms. However, many different sources are there but cost benefit analysis is employed for the maximization of the yield of enzyme.

3.4. Effect of different solvent on laccase production

Five different solvent sources like ethanol, methanol, formic acid, toluene and isopropanol were tested for extracellular laccase production in *Ganoderma* sp. Among them, ethanol supported the maximum laccase production (Fig. 6).Ethanol is an abundant agro-industrial by-product, cheaper and environmentally safer. Adiveppa and Kaliwal, (2015) reported that the ethanol supported maximum laccase production by *Marasmius* sp. BBKAV79 [33]. Sivakumar *et al.,* (2010) have showed the positive effect of isopropanol on laccase activity [21].

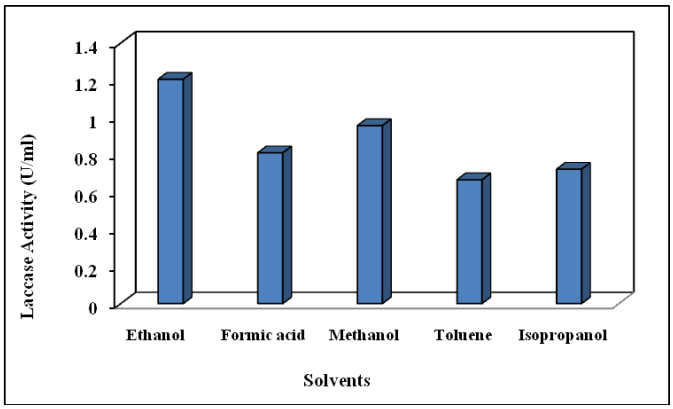

Fig. 6: Effect of solvent on laccase production

4. CONCLUSION

The findings of the present study clearly indicate that *Ganoderma* sp. is an efficient producer of laccase under submerged fermentation condition. YPD-Cu medium composition enhanced laccase production. Optimum pH and temperature for laccase production was 6.0 and 40 °C respectively. Starch and peptone were the most suitable carbon and nitrogen source for laccase production. Ethanol is best supporter for laccase production. Present study concluded that the effect of individual parameter can be best studied by using one variable at a time approach, to judge the effectiveness of each parameter on enzyme activity.

5. ACKNOWLEDGEMENT

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