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Efficiency of Paddy Residues as Substrates for Fungal Laccase Production

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ABSTRACT: Laccase is one of the key enzymes involved in lignin decomposition of forest litter and plays an important role in the carbon cycling in forest ecosystem. The ability of laccases to act on wide range of substrates has made them very important in many of the biotechnological applications like detoxification, decolourisation of industrial effluents, paper and pulp industries, textile industries, beverage processing and in bioremediation. Hence laccases have received a greater attention around the globe. Lignin is one of the most important structural components of the plant biomass. In the present study, laccase producing fungi were isolated from the forest litter of Kodagu and screened for their efficiency to produce laccase enzyme using paddy residues as substrate. The culture conditions were optimized with respect to pH, incubation temperature and incubation period for maximum laccase production by the isolates. *Mucor sp* was found to be a better producer of laccase enzyme in media supplemented with paddy husk at pH 5 in rotary condition on 6th day of incubation.

Keywords: litter, laccase, screening, growth optimization, *Mucor sp.*

I. INTRODUCTION

Soil microorganisms secrete numerous extracellular enzymes which play a key role in decomposition processes and secreted oxido-reductases such as Mn peroxidases, lignin peroxidases, and laccases, are involved in decomposition of lignin and phenols [1]. Laccase is a multi copper-containing polyphenol oxidase (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) belonging to the group of blue oxidases. Laccases are widely distributed in higher plants and fungi and more than 60 fungal strains, belonging to various classes such as Ascomycetes, Basidiomycetes and Deuteromycetes, have been demonstrated to produce laccase [2]. They are able to recognize the aromatic structure of the substrates and hydrolyse the phenolic hydroxyl groups into radical form and this initiate the breaking of the long chain lignin structure [3]. In fungal physiology, laccases are involved in plant pathogenesis, pigmentation, detoxification, lignin degradation and also in development of morphogenesis of fungi [4].

Interest in laccases has increased recently because of their potential use in the detoxification of pollutants and in bioremediation of phenolic compounds. These fungal enzymes can convert wood, plastic, paint, and jet fuel among other materials into nutrients [5]. The effective use of industrial laccases may be hindered by their non-reusability, and high sensitivity to denaturing agents. These undesirable restraints can be removed by use of modified or immobilized laccase. Many attempts have been made to improve the storage and operational stability of laccase, leading to lower cost and expansion of its biotechnological and environmental applications[6].Laccases are also used as catalysts for the manufacture of anticancer drugs and even as ingredients in cosmetics [7].

II. METHODOLOGY

A. Isolation and Screening of Fungi for laccase Enzyme Production

The litter samples collected from some selected forest areas and agricultural fields of Kodagu District, Karnataka State were serially diluted up to 10^{-6} dilution. 1ml each from 10^{-4} dilution was inoculated on petriplates containing potato dextrose agar (PDA) media supplemented with 0.04% guaiacol. In addition to this 0.01% (w/v) chloramphenicol was also added to the media to avoid bacterial growth and the pH of the medium was adjusted to 5.5 with 1 N HCL. The isolates showing reddish brown zones around the colony were considered to be laccase positive isolates [8]. The organism showing faster growth and wider zone was selected as potent strain. The strain was identified by Lactophenol cotton blue dye, observed under microscope and identified using standard manuals [9].

B. Confirmatory test for laccase producing fungi

All the isolated organisms were inoculated on petriplates containing PDA medium with tannic acid and observed for the development of brown colored precipitates and was assessed on daily basis [10].

C. Primary inoculum preparation

The isolated fungal strains were maintained on 2% (w/v) Malt Extract Agar slants at 4ºC and the fungi were activated at 26ºC. The malt extract broth of pH 5.8 was prepared. The mycelium were harvested with sterile 0.9% NaCl solution and then inoculated into 100 ml 2% malt extract broth Then it was incubated at 30˚C in a rotary shaker for 5 days. The inoculum was used for further enzyme production [11].

D. Optimization of Production conditions for laccase enzyme in submerged fermentation

Paddy husk and paddy straw containing enzyme production media was prepared. It consisted of Paddy straw or Paddy husk: 30g/L, Peptone:2g/L, Beef extract:2g/L, KH_2PO_4:3g/L, $MgSO_4$:0.5g/L, Vit B_1:0.02g/L, NaCl0.01g/L, $CaCl_2$:0.1g/L. The pH was adjusted to 5 and 7 respectively. Media was sterilized at 121˚C for 15min under 15 lbs. Each flask was inoculated with 5 ml of primary inoculum. The flasks were incubated under different fermentation conditions such as Rotary (165 rpm) and Stationary conditions at 26˚C. After the incubation, solids were separated by centrifuging at 6,000 rpm for 15 minutes. Supernatant was used for enzyme analysis The laccase activity was estimated on alternate days upto 12 days. All enzymatic analysis was carried out in duplicates [12].

E .Guaiacol assay method for determination of laccase enzyme activity

Guaiacol has been reported as efficient substrate for laccase assay. The intense brown colour development due to oxidation of guaiacol by laccase can be correlated to its activity often read at 465 nm. 0.2 ml of enzyme solution (0.2ml) was taken. Incubated in 4.0ml of 50 mM sodium acetate buffer (pH 4.5) containing 1.0mM guaiacol at 30˚C. The change in the absorbance due to oxidation of guaiacol in the reaction mixture was monitored at 465 nm (ε465= 12,100$M^{-1}cm^{-1}$). One unit of enzyme activity was defined as the amount of enzyme that oxidized 1µ mol guaiacol per minute under the standard conditions. Enzyme activity was calculated using the formula.

Enzyme activity, U/ml

$$= \frac{Absorbance/minute}{\varepsilon * d} \times \frac{Reaction\ volume}{Sample\ volume} \times 10^3$$

ε = Extinction coefficient for each substrate (cm^{-1} M^{-1})
d = Cuvette diameter

The total protein content of the extract was measured using bovine serum albumin (BSA) as standard by Lowry's Method [13].

III. RESULTS AND DISCUSSION

Morphologically 10 different fungal colonies showing reddish brown zones around themselves on PDA media containing 0.04% guaiacol were observed and considered to be laccase positive isolates. The isolates were identified based on its colony morphology as well as microscopic visualizations as *Cladosporium herbarum, Penicillium viridictum, Phanerochaete sp, Aspergillus fumigates, Cladosporium cladosporoidous, Trametes sp, Pycnosporus sp, Fusarium sp, Pleurotus sp* and *Mucor* sp respectively. Two potent isolates *Phanerochaete sp,* and *Mucor* sp showed fast growth and brown color precipitate within 3 days of incubation period. Other isolates took more than 6-7 days of incubation for appropriate growth and oxidation of substrate employed in the medium. Thus *Phanerochaete sp,* and *Mucor sp* were presumed to be the potent among all isolates and hence was used for further work. Kumar *et al.,* in 2011, had screened fungal species namely *Pleurotus, Fusarium, Agaricus, Penicillium, Trichoderma, Aspergillus, Beauveria, Verticillium, Paecilomyces* and *Metarhizium* for laccase activity by indicator plate method. The use of guaiacol and syringaldazine as an indicator of action for laccases provided for the rapid visual manifestation of laccase positives [14].

The optimization of growth conditions is essential because most of the extracellular enzymes prefer optimum environment conditions. Here the growth study was done with different pH, different substrate in rotary and stationary incubation condition. The potent organism was initially cultivated in enzyme production media containing paddy husk and paddy straw. The enzyme activity was measured every alternate day and continued until decrease in activity level was observed.

The *Phanerochaete sp.* showed maximum enzyme production in media supplemented with paddy husk at pH 5 in rotary condition on 6^{th} day of incubation. The enzyme activity was found to be 0.231 IU/ml. In case of media supplemented with Paddy straw, *Phanerochaete sp* showed maximum enzyme production of 0.215 IU/ml at pH 5 in rotary condition on 6^{th} day of incubation. This clearly indicated that paddy husk as substrate, pH 5, rotary condition and 6 days of incubation is optimum for laccase production by *Phanerochaete sp.*

Packiyam (2014), reported that the white rot fungi produced maximum levels of laccase between 5 to 9 days of incubation. He optimized the laccase enzyme production in *Pleurotus sp*. He reported that Laccase activity was 0.050 U/mg found in paddy straw supplement media. High laccase production was observed on 6^{th} day in *P. Sajorcaju*, 5^{th} day in *P. pulmonaris*. The pH optima of laccases are highly dependable on the substrate [15].

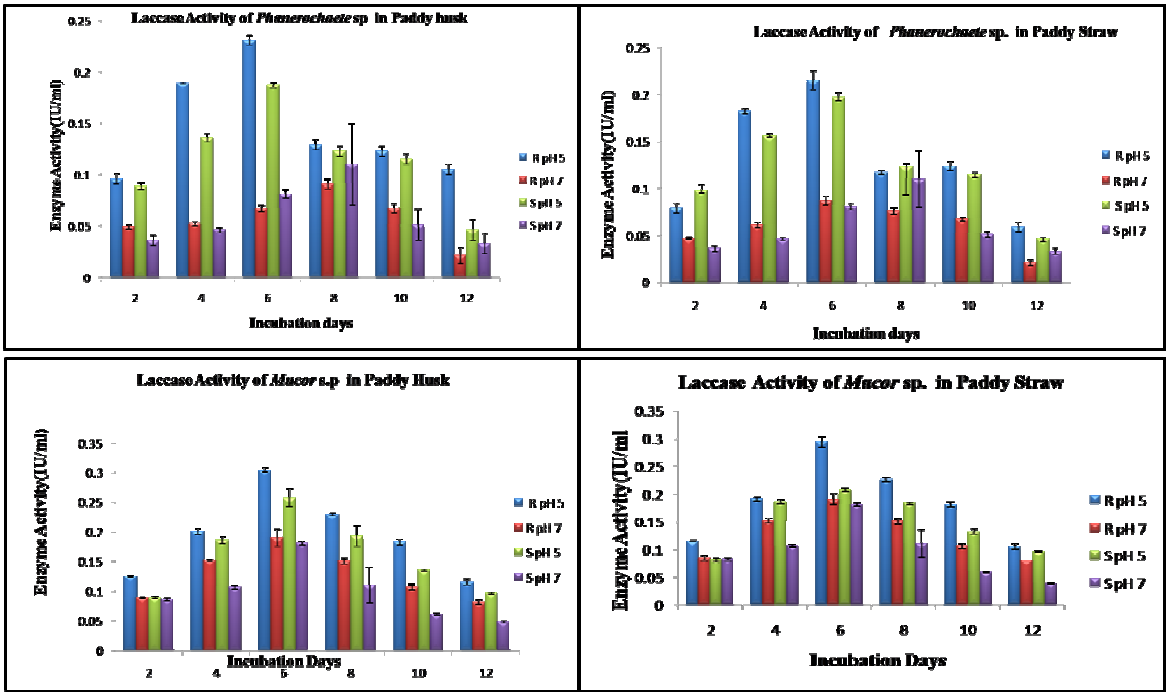

Fig. 1. Laccase Activity of different isolates in Paddy Straw and husk.

Note: The bars represent values in IU/ml ±SE (Standard Error). R pH 5=Enzyme activity at pH 5 under rotary condition; R pH 7 = Enzyme activity at pH 7 under rotary condition; S pH 5=Enzyme activity at pH 5 under stationary condition; S pH 7= Enzyme activity at pH 5 under stationary condition.

In the present study, laccase enzymatic activity was found to be higher than the study conducted and reported by Packiyam. The effect of temperature is limited in case of laccase production. The optimal temperature of laccase differs greatly from one strain to another. It has been found that 25°C is the optimal temperature for laccase production in presence of light, but, in case of dark, the optimal temperature is 30°C. The optimum temperature range for laccase production is between 25°C and 30°C. The *Mucor* sp. showed maximum enzyme production in media supplemented with paddy husk at pH 5 in rotary condition on 6th day of incubation. The enzyme activity was found to be 0.304 IU/ml. In case of media supplemented with Paddy straw, *Mucor* sp showed maximum enzyme production of 0.257 IU/ml at pH 5 in stationary condition on 6th day of incubation. This clearly indicated that paddy husk as substrate at pH 5 under rotary condition at a speed of 165 rpm and 6 days of incubation period is optimum for laccase production of *Mucor* sp.

Most of fungal cultures prefer a slightly acidic pH in the medium for growth and enzyme biosynthesis. Maximal formation of *Cladosporium werneckii* and *Auerobasidium pullulan* laccase took at pH 5.0 and pH 6.0 respectively. Fungal growth was relatively diminished as slight changes in pH affected the growth and hence the laccase production. The fungal laccase formation occurred at a narrow range of pH values, whereas low levels of enzyme were obtained at pH values below and above this value. This may be attributed to the fact that change in pH value may alter the three-dimensional structure of the enzymes [16]. Adejoye and Fasidi (2010) reported the *Schizophyllum commune* had its pH optimum at 5.5 [17]. Birhanli and Yesilada in 2013 studied the laccase enzymes production using various lignocellulosic wastes on submerged fermentation conditions. He reported that the maximum enzyme activity by *Coriolus versicolor* (0.22 IU/mL) and *Phanerochaete chrysosporium* (0.24I U/mL) in rice bran supplemented media [18]. Elisashvili *et al* (2008) and Sivakumar *et al* (2010), reported that maximum laccase production was obtained at the 7th and 10th day of incubation in case of *Lentinus edodes* and *Ganoderma* sp, respectively [19]. Packiyam in 2014, reported that the white rot fungi produced maximum levels of ligninases, lipase, Mn peroxidase and laccase between 5 to 9 days of incubation. High laccase production was observed on 6th day in *P. Sajorcaju*, 5th day in *P. pulmonaris*. The pH optima of laccases are highly dependable on the substrate. When using ABTS as substrate the pH optima are more acidic and are found in the range 3 to 5, Kunamneni *et al*., (2007) found that the optimal temperature for fruiting body formation and laccase production is 25°C in the presence of light, but 30°C for laccase production when the cultures are incubated in the dark.

In general the fungi were cultivated at temperatures between 25°C and 30°C for optimal laccase production [20]. The protein content in Paddy husk inoculated with *Mucor sp.* was found to be decreasing on each day of 12 days incubation.

IV. CONCLUSION

The present study throws an open light to the treasure of Laccase enzyme producing fungal resources which otherwise go unnoticed. Study clearly indicates that the fungus *Mucor sp and Phanerochaete* sp isolated from litter samples have the ability to produce laccase enzyme. Use of cheap, easily available substrates such as paddy straw and paddy husk can be used for culturing these fungi since they provide nutrition to fungi. Media optimization and use of appropriate inducers could bring additional benefits of higher production with expenditure of minimum resources. *Mucor sp* was found to be a better producer of laccase enzyme as compared to *Phanerochaete sp* under different fermentation condition. Hence these isolates can be exploited for the production of industrially important laccase enzymes.

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