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CHARACTERIZATION OF LIGNINOLYTIC ENZYMES AND DECOLOURIZATION OF SELECTED TEXTILE DYES FROM THE BLUSHING BRACKET MUSHROOM, *DAEDALEOPSIS CONFRAGOSA*

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ABSTRACT: The effect of a wide range of culture conditions on the production of laccase (Lac), Lignin peroxidase (Lip) and Manganese peroxidases (MnP) by *Daedaleopsis confragosa* was studied. A ligninolytic enzyme-producing bracket mushroom, *Daedaleopsis confragosa* was isolated from tree trunk from Karwar coastal region, Karnataka, India and subjected to production and dye decolorization study. Ligninolytic enzyme production was done by Submerged Fermentation (SmF). Various process parameters like different pH, temperature, carbon, nitrogen sources, and metal ions were investigated on Laccase (Lac), Lignin peroxidase (LiP) and Manganese peroxidizes (MnP) production in the fermentation process. The study revealed that the Lac, Lip and MnP highest activity was observed at pH 6.0, 5.5, and 7.2 respectively. Lac, Lip, and MnP showed highest activity at temperature 45°C, 40°C and 40°C respectively. Glucose as carbon source and modified PDB (Potato dextrose broth +2% saw dust + 1% yeast extract) as nitrogen source support maximum laccase activity. Starch as carbon source and modified PDB as nitrogen source enhanced the highest activity of Lignin peroxidase and Manganese peroxidases, respectively. Laccase (Lac), Lignin peroxidase (LiP), and Manganese peroxidases (MnP) activity were highest with copper ion supplement. Further textile dye decolorization was performed with Red M8B, Green HE4B, Navy blue HER, Orange HE2R, were decolorized at a range of 80-95%.

INTRODUCTION: Laccases, peroxidases (including lignin peroxidases, manganese peroxidases, and manganese-independent peroxidases), and H₂O₂-generating oxidases are components of the lignin-degrading enzyme system¹. So far, wood-rotting fungi, such as white rot and soft rot fungi are the only organisms known to be capable of extensively degrading lignin².

There has been great interest in using fungal laccases and peroxidases for biotechnological processes due to their chemical and catalytic features^{3, 4}. Investigations of the use of wood-rotting fungi and their ligninolytic enzymes in bio pulping processes in paper-making industries could lead to ways to reduce the energy and chemical requirements of those processes. A biobleaching process requires substantial enzyme activities at an alkaline pH and a high temperature. Also, several other potential applications have been suggested for producers of ligninolytic enzymes⁵.

Laccases (EC 1.10.3.2; benzenediol: oxygen oxidoreductases) are for the most part extracellular copper-containing glycoproteins with molecular

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weights between 60,000 and 80,000. Lignin peroxidases (EC 1.11.1.14; diarylpropane:oxygen, hydrogen peroxide oxidoreductases; molecular weights, 38,000 to 43,000) and manganese peroxidases (EC 1.11.1.13; Mn(II): H₂O₂ oxidoreductases; molecular weights, 43,000 to 49,000) are glycoproteins containing one protoporphyrin IX as a prosthetic group⁶. The reactions catalyzed by laccases and peroxidases are very similar^{7, 8, 9, 10}. Both types of enzymes oxidize phenolic compounds and aromatic amines via one-electron oxidations, which creates radicals. Besides differences in the prosthetic groups, the laccases also differ from the peroxidases by generally having a lower oxidation potential^{11, 12}.

Many producers of laccase (e.g., *Ceriporiopsis subvermispota*¹³, *Coriolus versicolor*¹⁴ and *Panus tigrinus*¹⁵, *Marasmius* sp.,¹⁶ *Coprinus* sp.¹⁷, *Ganoderma* sp.¹⁸ and producers of lignin peroxidases and manganese peroxidases (e.g., *Phanerochaete chrysosporium* and *Bjerkandera adusta*¹⁹ secrete isoenzymes which differ in stability and catalytic features²⁰. The extracellular peroxidases isolated from *Coprinus cinereus*²¹ and

*Arthromyces ramosus*²² differ from lignin peroxidases and manganese peroxidases by having broader substrate specificities and in the architecture of their active sites.

We recently discovered that agaricomycetes *Daedaleopsis confragosa* produce laccases, Lignin peroxidases, and Manganese peroxidases and the purpose of this study was to report the production in submerged fermentation and to perform the decolorization study.

MATERIALS AND METHODS:

Collection of Sample and Isolation of Fungus: A ligninolytic enzyme-producing bracket mushroom, mycelia were isolated from tree trunk from Karwar coastal region, Karnataka, India. For the isolation of *Daedaleopsis confragosa* **Fig. 1**, a young fruiting body was briefly washed with sterile water and 1% sodium hypochlorite (1min) then fruiting body was cut into small pieces (2-5 mm long) in a bottle containing sterile water. The solution was homogenized and a small aliquot was inoculated onto potato dextrose agar medium.



FIG. 1: LIGNINOLYTIC ENZYMES PRODUCING BRACKET MUSHROOM, DAEDALEOPSIS CONFRAGOSA

Qualitative Screening of Ligninolytic Enzyme on Solid Media: Inoculation of mycelium onto PDA plates containing 0.02% Guaiacol, 1mM ABTS, 0.5% Tannic acid, and 0.1% Syringaldazine as indicator compound and it was incubated at 30 °C for 5 days. For guaiacol, tannic acid and syringaldazine the formation of reddish-brown halo zone in plates indicated a positive ligninolytic enzyme secretion and ABTS the formation of purple halo zone in plates indicated a positive ligninolytic enzyme secretion assay method.

Extracellular Ligninolytic Enzymes Activity:

Laccase Assay: The culture supernatant was added with 1 ml of guaiacol and 100 mM Phosphate

buffer (pH 5.5). The reaction mixture was incubated at 40 °C for 30 min. The color change was measured using spectroscopy at 460 nm. One unit of laccase activity can be defined as amount of enzyme required to hydrolyze guaiacol during incubation period²³.

Lignin Peroxidase Assay: To 1 ml of culture supernatant, 2 mM vetryl alcohol, 0.27 mM H₂O₂ and 10 mM tartrate buffer (pH 3.0) were added and incubated for 30 min. The color change was read at 310 nm. The enzyme activity can be measured by amount of enzyme required to convert one unit of vetryl alcohol into vetryl aldehyde during the incubation period²⁴.

Manganese Peroxidase Assay: The MnP activity was determined as described by Kuwahara *et al.*, (25) using a solution of phenol red (1 g L^{-1}) as substrate. For the reaction we used 100 μL phenol red, 200 μL sodium lactate, 300 μL BSA (0.5 %), 50 μL manganese sulfate II, 100 μL hydrogen peroxide in sodium succinate buffer (2 M, pH 4.5), 100 μL fungal extract and 150 μL distilled water. The reaction was incubated at 37 °C for 15 min. The reaction was stopped with 40 μL of sodium hydroxide (2M). The absorbance was measured at 610 nm²⁶.

Optimization of Ligninolytic Enzymes in Submerged Fermentation:

Effect of pH and Temperature on Ligninolytic Enzymes Production: The effect of pH was studied by adjusting the pH of the production media to different pHs ranging from 4.5, 5.0, 5.5, 6.0, 6.5, 7.2, 8.0, 8.5, 9.0 on ligninolytic enzyme production in production media were done by inoculating *Daedaleopsis confragosa* and incubated the flasks in incubator and assay were done as per ligninolytic assay method. Similarly to study the effect of temperature, the *Daedaleopsis confragosa* was inoculated in the production media at different temperatures ranging from 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C and 55 °C and assay was done as per ligninolytic enzymes assay method.

Effect of Carbon and Nitrogen Sources on Ligninolytic Enzyme Production: Different carbon sources namely glucose, sucrose, starch, lactose, mannitol and maltose were added to production media at the concentration of 2%. The flasks were inoculated with well-grown *Daedaleopsis confragosa* discs from PDA plates and the flasks were incubated at 40 °C and assay were done as ligninolytic assay method. Similarly different nitrogen sources namely Trypton, Peptone, Yeast extract, Soya bean meal, GYMP (Glucose yeast extract malt peptone) and Sodium carbonate were added to production media at the concentration of 2% and the flasks were inoculated with well-grown *Daedaleopsis confragosa* discs from PDA plates and the flasks were incubated at 40 °C and assay were done as ligninolytic assay method.

Effect of Metal Ions on Ligninolytic Enzyme Production: The effect of metal ions on the

activity of the enzyme was studied to determine nature of active site of enzyme. During this study following salts of ions were taken at the rate of 50 mM/ml and 100 mM/ml of reaction mixture. The ions used were Ag^+ (AgNO_3), Mg^{+2} (MgSO_4), Mn^{+2} (MnSO_4), Fe^{+2} (FeSO_4), Cu^{+2} (CuSO_4), Zn^{+2} (ZnSO_4) and Hg^{+2} (HgCl_2).

Decolourization of Selected Textile Dyes:

Decolourization experiment was conducted in 250 ml Erlenmeyer flask containing 100 ml of Mod. PDB medium. The *Daedaleopsis confragosa* was tested for its ability to decolorize selected textile dye over a period of 24 h. The final concentrations of the dye in the medium without inoculation with *Daedaleopsis confragosa* was considered as a control. The extent of decolorization was recorded as residual color. The dyes (50 mg l^{-1}) were monitored at their absorbance maxima at 620 nm²³.

Decolourization Initial absorbance (%) = $\frac{\text{Final absorbance} - \text{Initial absorbance}}{\text{Initial absorbance}} \times 100$

The effect of dye decolorization was determined by the decrease in absorbance under the maximum wavelength of the dye. The efficiency of decolorization was expressed in terms of percentage²³.

RESULTS AND DISCUSSION:

Qualitative Screening of Ligninolytic Enzyme on Solid Media: Isolated fungal was maintained in PDA medium. They were screened for ligninolytic enzyme production using four indicators namely ABTS, Guaiacol, Syringaldazine, and Tannic acid. Ligninase is a very potent enzyme with ability to act on a number of substrates. Due to which, it gains high industrial importance. The isolate was inoculated in potato dextrose agar plate which is containing 0.02% guaiacol, 1mM ABTS, 0.5% Tannic acid and 0.1% syringaldazine as indicator compound and it was incubated at 30 °C for 5 days. In the presence of guaiacol, Tannic acid and the syringaldazine intense reddish-brown color was produced in the medium around the fungal colonies and was taken as the positive reaction for the production of ligninolytic enzymes. In the presence of ABTS purple-color in the presence of fungal colonies and was taken as the positive reaction for the production of ligninolytic enzymes¹⁶.

Effect of pH and Temperature on Ligninolytic Enzyme Production: The pH optima for ligninase production by white-rot fungi are highly dependent on chemical composition of the substrates and fermentation media^{27, 28}. The result revealed that the Laccase (Lac), Lignin peroxidase (LiP) and Manganese peroxidases (MnP) was observed highest activity at pH 6.0 (0.880 U/ml), 5.5 (30.43 U/ml) and 7.2 (0.180 U/ml) respectively **Fig. 2**. Similar to our findings, maximum LiP, and MnP activities were produced by *Pleurotus ostreatus* in the pH range 4.0 to 5.0 at 25 °C. The optimum ligninase production by *Coriolus hirsutus* and *Trametes villosa* CCB176 has been reported at pH 4.0 and 5.0, respectively^{29, 30}.

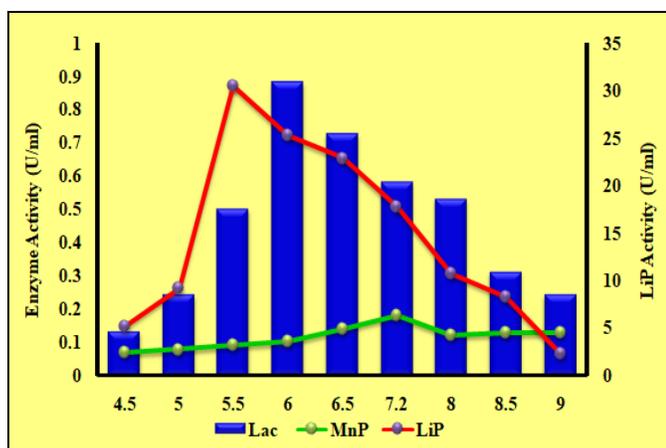


FIG. 2: EFFECT OF pH ON LIGNINOLYTIC ENZYMES ACTIVITY

The incubation temperature showed a considerable impact on ligninolytic enzyme production. Laccase (Lac), Lignin peroxidase (LiP) and Manganese peroxidases (MnP) showed maximum activity at temperature 45 °C (0.882 U/ml), 40 °C (30.43 U/ml) and 40 °C (0.145 U/ml) respectively **Fig. 3**.

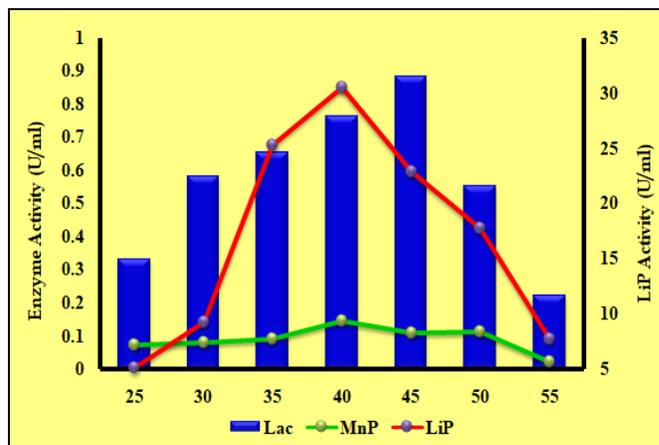


FIG. 3: EFFECT OF TEMPERATURE ON LIGNINOLYTIC ENZYMES ACTIVITY

Coriolus hirsutus has also been found to excrete a considerable amount of laccase and Manganese peroxidases at 28 °C³¹. A significant influence of incubation temperature on ligninolytic enzymes of *Pleurotus* sp. and *Dichomitus squalens* temperature ranging from 25 °C to 30 °C were found optimum for ligninase production^{32, 33, 34, 35}.

Effect of Carbon and Nitrogen Sources on Ligninolytic Enzyme Production: Optimization of the cultivation conditions for ligninolytic enzyme production is extensively explored with selected *Daedaleopsis confragosa*. The results showed that the glucose (0.812 U/ml) as carbon source and modified PDB (Potato dextrose broth +2% saw dust + 1% yeast extract) (1.120 U/ml) as nitrogen source supports maximum laccase activity. Starch (30.23 U/ml and 0.162 U/ml) as carbon source and modified PDB (29.23 U/ml and 0.182 U/ml) as nitrogen source enhanced the highest activity of Lignin peroxidase and Manganese peroxidases respectively **Fig. 4** and **Fig. 5**.

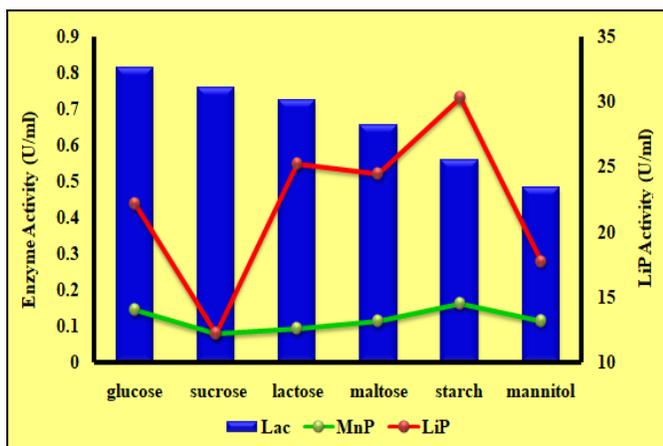


FIG. 4: EFFECT OF CARBON SOURCES LIGNINOLYTIC ENZYMES ACTIVITY

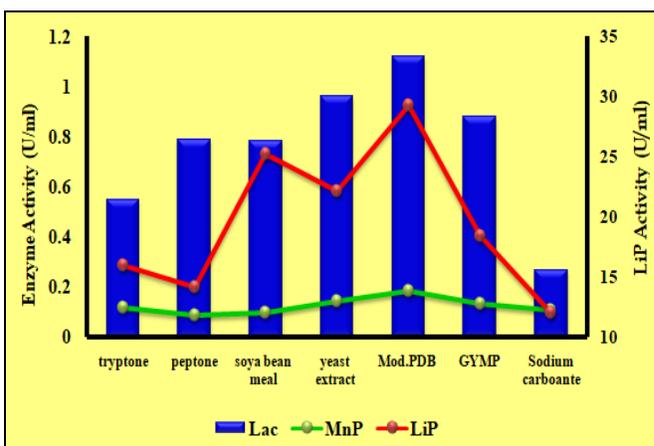


FIG. 5: EFFECT OF NITROGEN SOURCES LIGNINOLYTIC ENZYMES ACTIVITY

Significant laccase secretion by *Trametes pubescens* started when the glucose concentration in the growth medium reached a certain low, critical concentration³⁶. The highest laccase yields by using a combination of glucose and starch as carbon and yeast extract as a nitrogen source by *Coriolus versicolor*³⁷.

The enzyme profiles varied with different carbon and nitrogen source combinations. The source and concentration of carbon and nitrogen are the powerful factors regulating the synthesis of ligninolytic enzymes by white-rot fungi³⁸. Peptone turned out to be the best nitrogen source for laccase and MnP accumulation by *Coriolopsis gallica*³⁹. Peptone enhanced the production of ligninolytic enzymes by *Pleurotus ostreatus*⁴⁰ and *Polyporus sanguineus*⁴¹.

Effect of Metal Ion Ligninolytic Enzyme Production: The interaction of metals with extracellular laccase was particularly important for a better understanding of the biotechnological processes of xenobiotic degradation. Therefore, the stability of ligninolytic activity against several metal compounds was tested Laccase (Lac), Lignin peroxidase (LiP) and Manganese peroxidases (MnP) activity was highest with copper ion (5mM) (0.924 U/ml, 31.2 U/ml, and 0.165 U/ml, respectively) supplement **Fig. 6**.

Previous reports are showing that the addition of 100 mM CuSO₄ during the exponential growth phase of the fungus led to a remarkably increased laccase production³⁶. Supplementation of CuSO₄ at 100 mM concentration yielded high amounts of laccase (175 IU/ml) at an incubation period of 5 days⁴².

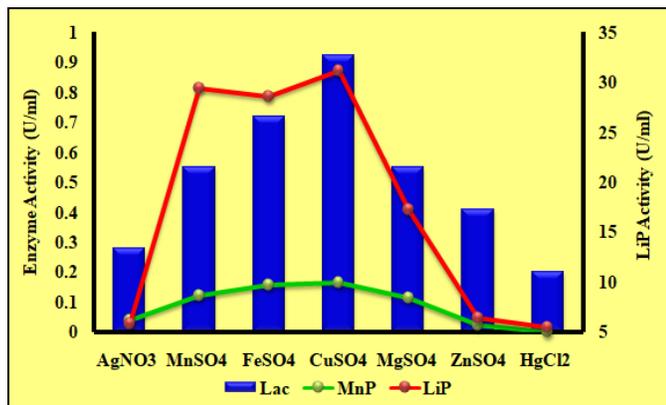


FIG. 6: EFFECT OF METAL IONS (5mM) ON LIGNINOLYTIC ENZYMES ACTIVITY

Decolourization of Textile Dyes by *Daedaleopsis confragosa*: The isolated *Daedaleopsis confragosa* was tested for their ability to decolorize the selected dyes i.e., Navy blue HER, Orange HE2R, Red M8 B, and Green HE4BD by 75.82%, 70.40%, 80.05% and 90.08% within 96 h respectively. A similar result was reported by Vantamuri and Kaliwal, (2016) as Navy blue HER, Green HE4BD and Orange HE2R by 72.47%, 75.31.05% and 76.58% within 96 h respectively **Fig. 7** and **Fig. 8**²³. The *Pseudomonas putida* was tested for their dye decolorizing ability against synthetic dyes and industrial effluents⁴³. The white-rot fungi *Trametes hirsute* and *Pleurotus florida* for their dye decolorising ability against three reactive dyes Blue CA, Black B133 and Corazol violet SR⁴⁴. *Pleurotus sajor-caju* under suspension culture completely decolorized several phenolic azo dyes⁴⁵.

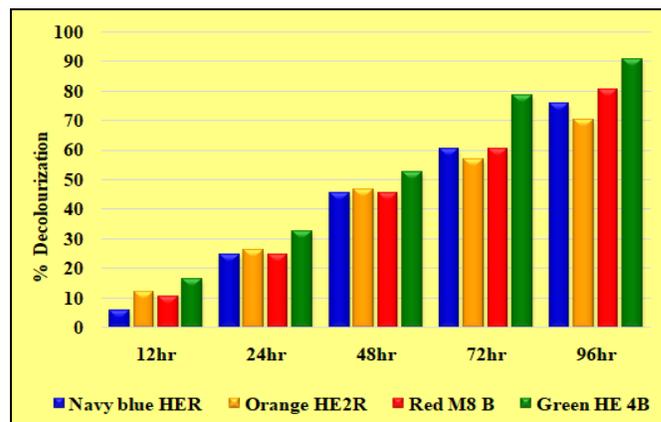


FIG. 7: DECOLOURIZATION OF SELECTED TEXTILE DYES

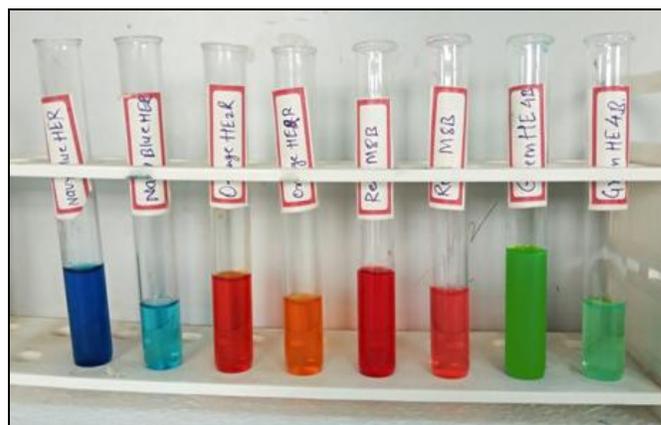


FIG. 8: DECOLOURIZATION SELECTED TEXTILE DYES BY DAEDALEOPSIS CONFRAGOSA

CONCLUSION: In conclusion, the literature data and the results obtained in this work show that the effect of carbon and nitrogen sources depends on the fungal strain and nature of the compound

tested. *Daedaleopsis confragosa* was found to be a new fungal isolate that exhibited high decolorization activity and also exhibited thermos tolerant ligninolytic enzymes. In addition, these major components of nutrition media are factors not only determining the expression of fungal enzyme activity but also the ratio of individual enzymes in enzyme complexes. Further investigation required revealing the structure of the produced enzyme with respect to the inducer structure and mechanism will contribute to several industrial applications. The promisingly high activities of MnP and LiP suggest the possibility of commercialization of the production process.

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