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ORIGINAL RESEARCH PAPER

# Isolation, production and laccase isozyme using different lignocellulosic waste under SmF and SSF condition

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**Abstract**

## Technology Area

*This technology falls under Industrial and Environmental Biotechnology, specifically focusing on enzyme technology, microbial bioprocess optimization, and agro-waste valorization for sustainable enzyme production.*

## Technology

*This technology involves the isolation and optimization of laccase-producing basidiomycetes for enhanced enzyme production using both submerged fermentation (SmF) and solid-state fermentation (SSF). The process utilizes copper sulphate as an inducer to significantly boost enzyme yield and employs agro-industrial waste (e.g., pomegranate peel) as a cost-effective substrate for enzyme production. The approach also enables the generation of multiple laccase isozymes depending on the substrate, enhancing its application versatility.*

## Potential Market / Customers

*Paper and pulp industries (biobleaching), Textile industries (dye degradation), Bioremediation and wastewater treatment plants, Biofuel and biorefinery industries, Food processing industries, Pharmaceutical and cosmetic industries, Technology Valuation (Commercial Potential & Value Proposition)*

## Technology Valuation (Commercial Potential & Value Proposition)

*The technology demonstrates strong commercial potential due to: Cost-effective production using agro-waste substrates , High enzyme yield through induction strategies (90-fold increase), Eco-friendly and sustainable process suitable for green industries, Versatility of applications across multiple industrial sectors, Scalability potential for industrial enzyme production*

**Keywords** Ganoderma, Laccase, Isozyme,Basidiomycetes, Co-cultivation

## Introduction

White rot fungi are responsible for the destruction and decay of polysaccharides and lignin (Kuhad et. al., 1997). They are the only known microorganisms that have evolved complex enzymatic systems that enable them to degrade lignin (Garzillo et al., 1998). White-rot fungi are currently of great interest, as many species in this group have been described as producers of lignolytic enzymes that are involved in the natural delignification of wood (Archibald & Roy, 1992).

Some of the more important enzymes responsible for lignin degradation in nature include lignin peroxidase (diaylpropane: oxygen, hydrogen peroxide oxidoreductase; EC 1.11.1.14), manganese peroxidase (Mn(II): hydrogen peroxide oxidoreductase; EC 1.11.1.13) and laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2.) (Call &Mücke, 1997; Heinzkill et al., 1998).

Many efforts have been made to utilize enzymes for the degradation of lignin in the pulp and paper industry (Call &Mücke, 1997). One enzyme known to play a major role in natural delignification is laccase (EC 1.10.3.2; benzenediol:oxygen oxidoreductase) (Call &Mücke, 1997). Among the large blue copper containing enzymes, laccase is the most widely distributed (Leontievsky et al., 1997) and occurs in various plants and fungi (Sharma and Kuhad, 2008).

In the fungi, Deutromycetes, Ascomycetes and a wide range of Basidiomycetes are known producers of laccases, which are particularly abundant in many lignin-degrading white-rot fungi (Leontievsky et al., 1997; Thurston, 1994).

Laccases are mostly extracellular glyco-proteins (Heinzkill et al., 1998) and are multinuclear enzymes (Gayazov&Rodakiewicz-Nowak, 1996) with 60 and 80 kDa molecular weights (Heinzkill et al., 1998; Leontievsky et al., 1997; Thurston, 1994). Most monomeric laccase molecules contain four copper atoms in their structure that can be classified in three groups using UV/visible and electron paramagnetic resonance (EPR) spectroscopy (Thurston, 1994; Leontievsky et al., 1997).

*Ganoderma* sp., a ubiquitous white-rot basidiomycete, has long been used as a traditional herbal medicine in much of Asia and has been found to produce antitumor and hypoglycemic polysaccharides and immunomodulatory proteins, as well as bioactive oxygenated triterpenoids (Shankar et. al. 2024).

Laccases are widely distributed in higher plants, bacteria, archaea, fungi, and

insects. In plants, laccase plays a role in lignifications whereas in fungi it has been implicated in delignification, sporulation, pigment production, fruiting body formation, and plant pathogenesis (Galhaup et al. 2002; Sharma and Kuhad2008).

Laccase production by basidiomycetes under submerged fermentation (SmF) has been reported extensively but reports on solid state fermentation (SSF) are scanty (Vasdev and Kuhad 1994; Palmieri et al. 2003). Recently, SSF has emerged as a major thrust area in producing enzymes because of considerable economic potentials offered in terms of high volumetric productivity, low capital costs, reduced energy requirement and simple fermentation media (Kapoor et al. 2000; Tellez et al. 2008).

Fungi are grown optimally in a medium at pH 5), however, most studies show that pH between 4.5 and 6.0 is suitable for enzyme production (Thurston, 1994).

It has been 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 (Thurston, 1994). In general the fungi were cultivated at temperatures between 25 °C and 30 °C for optimal laccase production (Arora & Gill, 2000; Pointing et al., 2001). When cultivated at temperatures higher than 30 °C the activity of ligninolytic enzymes was reduced (Sharma et. al. 2005).

Laccase production has been found to be highly dependent on the conditions for cultivation of the fungus (Heinzkill et al., 1998) and media supporting high biomass did not necessarily support high laccase yields. Laccases were generally produced in low concentrations by laccase producing fungi, but higher concentrations were obtainable with the addition of various supplements to media (Sharma et. al. 2005). Low concentration of Cu+2 to the cultivation media increases the laccase production 50 times in comparison to basal medium (Galhaup and Haltrich, 2001).

Many laccase producing fungi secrete isoforms of the same enzyme (Leontievsky et al., 1997). These isozymes have been found to originate from the same or different genes encoding for the laccase enzyme (Archibald et al., 1997). The number of isozymes present differ between species and also within species depending on whether they are induced or non-induced (Kumar et. al. 2021). They can differ markedly in their stability, optimal pH and temperature and affinity for different substrates (Kumar et. al. 2021; Heinzkill et al., 1998). Furthermore, these different isozymes can have different roles in the physiology of different species or in the same species under different conditions

(Kumar et. al. 2021). Various laccase encoding gene sequences have been reported from a range of ligninolytic fungi (Jain et. al., 2020).

**Materials and Methods**

**Chemicals and Reagents**

All the chemicals used in this work were of high analytical grade. Imidazole used in the preparation of lysis buffer was purchased from Sigma-Aldrich, India. The chemicals Malt extract, Potassium dihydrogen phosphate ($KH_2PO_4$), Magnesium sulfateheptahydrate ($MgSO_4.7H_2O$), Calcium nitrate tetrahydrate $Ca(NO_3)_2.4H_2O$, Agar, Sodium chloride (NaCl), Ammonium sulfate $(NH_4)_2SO_4$), Calcium chloride ($CaCl_2$), Dibasic sodium phosphate ($Na_2HPO_4$), Citric acid ($C_6H_8O_7$), Guaiacol, Copper sulphate ($CuSO_4$), Sodium potassium tartrate, Folin, Pottasium hydrogen phosphate ($K_2HPO_4$), Tris Cl were purchased from Hi-Media and Merck. Hi-Media, India. All the glasswares were purchased from Borosil and Schott Duran, Germany.

The Fungal samples for the study were collected from Tilyar lake, Rohtak , Sampla, Bahadurgarh which included the wood dust, wood pieces of various sizes, bark and other plant portions. The samples were collected in sterile plastic bags and were sealed and brought to the lab aseptically for further processing.

The fruiting body of fungus growing on the bark of a tree was removed and thoroughly washed under running tap water. Fruiting body was cut into small pieces (3 mm X 3 mm) and surface sterilized with 0.1% $Hg_2Cl_2$ (w/v) for 1 min followed by 75% ethanol washing (Sharma et. al. 2025). Thereafter, it was rinsed with sterile distilled water under a biosafety hood to maintain aseptic conditions. The fruiting body was then dissected with the help of sterilized scalpel to get the basidiospores, which were inoculated on Malt extract agar (MEA) plates. The plates were incubated at 30°C in a BOD incubator. After incubation, the fungal isolate was subcultured and maintained on the MEA with the following composition: Malt extract- 20 g/L, Calcium nitrate tetrahydrate- 0.5 g/L, Potassium dihydrogen phosphate (KH2PO4) - 0.5 g/L, Magnesium sulphateheptahydrate (MgSO4.7H2O)- 0.5 g/L, Agar- 20g/L, pH- 5.2.to obtain pure culture (Vasdev and Kuhad, 1994; Sharma et al. 2005). Pure cultures were maintained at 37°C.

*Screening for laccase enzyme production*

Screening was performed by using plate assay method. The fungal carpet edges of

master plate were inoculated on MEA plates and incubated for 8-9 days at 30°C. The fungal plates were flooded with guaiacol. It acts as substrate for laccase and observed the plates for the development of reddish brown color compared to uninoculated agar plates.

*Production and optimization of laccase enzyme in submerged condition*

Each Erlenmeyer 250 ml flask having 25ml of sterile malt extract broth (MEB) containing (g/l) Malt extract 20.0; Ca(NO3)2.4H2O 0.5; MgSO4.7H2O 0.5, KH2PO4 0.5; Agar 20.0, pH 5.2 was inoculated with 4 discs obtained from the periphery of 4-5 days old culture of basidiomycetous fungus. The flasks were incubated under static cultivation conditions at 30°C for 14 days.

*Effect of metal ions on laccase production*

*Ganoderma* sp. Laccase production medium was further added using different metal ions such as $ZnSO_4$, $FeSO_4$, and $CuSO_4$ – all set at predefined concentration of 5mM to induce the production of laccase.

*Analytical procedures*

Guaiacol was used as substrate for assaying laccase activity. Laccase activity assayed according to modified method described by Vasdev and Kuhad (1994). 200 µL of culture supernatant (enzyme) was added to 1800 µL of guaiacol solution (10 mMguaiacol in Citrate-phosphate buffer, pH 5.2). After vortexing for few seconds at room temperature, incubate at 30°C for 30 mins, the absorbance was measured at 470 nm. The boiled culture supernatant was used as a control. One unit of laccase was defined as the change in absorbance of 0.01/ml/min at 470 nm.

*Co-cultivation under SSF*

*Inoculum Preparation*

Primary innocula was prepared by inoculating the flask with 4 discs obtained from the periphery of 4 day old culture of basidiomycetous fungus in Erlenmeyer flasks (250 ml) containing basal media. The 4-5 days old fungal culture was inoculated in 6 flasks, containing 25 ml basal media which were incubated at 30°C for 5 days at shakes flask conditions.

*Culture conditions*

Co-culture experiment was carried out in 250 ml Erlenmeyer flask, containing 10g of wheat bran and 1:1of kitchen waste, pomegranate peels waste and eucalyptus leaves waste with wheat bran (washed and dried and milled to 0.5-1.0 mm particle size). The wheat bran and wastes were moistened with mineral salt solution, containing (g/l): Malt extract 10.0, $KH_2PO_4$ 0.5, $MgSO_4.7H_2O$ 0.5, Ca

$(NO_3)_2.4H_2O$ 0.5, pH 5.2 in 1:5 ratio of solid substrate-to-liquid. The flasks were autoclaved at 15 psi for 20 minutes. The flasks were inoculated with pellets of fungal cultures and incubated at 30°C under static condition. The samples were taken after every 48 h starting from 4th day, vortexed after adding a significant volume of C.P buffer pH 5.2. Then sample was squeezed using muslin cloth and the supernatant was centrifuged at 4ºC and 6000g. The supernatant was used for enzyme assay for the estimation of Laccase, Xylanase, Protease, CMCase, FPase, and β- glucosidase activity.

*Analytical procedures*

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Xylanase, CMCase and FPase activity was measured using oat spelt xylan (0.6%) (Bailey et al. 1992), CMC (2%) and 1X6 cm filter paper strip. The release of reducing sugars in 10 min at 50°C, pH 6.2 (0.05 M citrate buffer) was measured as sugar equivalents using the dinitrosalicylic acid method (Miller 1959) at 540 nm. One unit of enzyme activity is defined as the amount of enzyme liberating 1 µmol of respective sugar per min.

β-glucosidase activity was assayed with 0.1% pNPG. The release of pNP- was estimated after the addition of 10% sodium carbonate at 400 nm.

Protease assay was assayed at 37ºC. In this assay, casein acts as a substrate. When the protease digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin's reagent primarily reacts with free tyrosine to produce a blue colored chromophore, which is quantifiable and measured as an absorbance value on the spectrophotometer. One unit of protease activity was defined as the amount of enzyme that resulted in the release of 1 µg of amino acid equivalent to tyrosine per minute.

The Native PAGE was carried out in a Biorad Mini-PROTEAN gel apparatus. Appropriate amount of the protein sample was mixed with the sample buffer. The samples were loaded in the gel and was

stained by guaiacol solution (10 mMguaiacol in Citrate-phosphate buffer, pH 5.2).

*Stock solutions for Native PAGE*

Acrylamide 29% (w/v) and 1% (w/v) N, N' methylene bis-acrylamide was dissolved in warm distilled water. The pH was checked to be 7.0 and stored in dark bottles at 4ºC

Resolving gel buffer (1.5M Tris, pH 8.8) was prepared by dissolving 91g Tris in 300 ml distilled water. The pH of the solution was adjusted to 8.8 using concentrated HCL and the volume was build up to 500 ml with distilled water. The solution was stored at 4ºC.Stacking gel buffer (0.5M Tris, pH 6.8) was prepared by dissolving 6.05g Tris in 40ml distilled water.

**Result**

**Isolation and screening of laccase producingwhite rot fungi**

A total of five basidiomycetous white rot fungi collected from different places. The fruiting body of basidiomycetous fungus was found to grow laterally like a shelf on the bark of tree (Fig. 1). Texture was soft and fleshy, colour of internal tissues was white to pale brown. All the morphological observations suggested that the fungus belongs to genus *Ganoderma*.

**Fig 1.**Different Basidiomycetes: Fruiting bodies of *Ganoderma*sp

**Primary screening of the fungus to produce laccase**

Basidiomycetous fungal isolate when grown on malt extract agar and flooded with guaiacol, a reddish brown color was observed due to the oxidation of guaiacol by the laccase. Out of 5, one was isolated successfully confirmed as laccase producer (Fig. 2A & Fig. 2B).

A

B

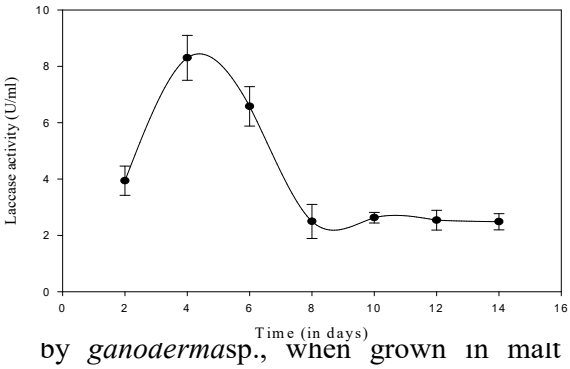
**Fig 2. A.** *Ganoderma* mycelia & Qualitative assay using Guaiacol substrate **B.** Pellets of fungal culture

***Production of laccase enzyme in submerged condition***

Laccase assay was performed with guaiacol as a substrate. Maximum activity was reported on 4th day after inoculation, i.e. 8.30U/ml (Fig. 3).

***Effect of metal ions***

The effect of metal ions was studied using different metal ions such as $ZnSO_4$, $FeSO_4$, and $CuSO_4$ at predefined concentration of 5mM to induce the production of laccase. Copper sulphate was found to be most potent inducer for laccase production, i.e., 753 U/ml on 12th day. Whereas, other metal ion i.e., $ZnSO_4$and$FeSO_4$ have no significant effect on laccase production (Fig. 4 & Fig. 5).




by *ganoderma*sp., when grown in malt extract broth (MEB) at 30ºC under static cultivation conditions

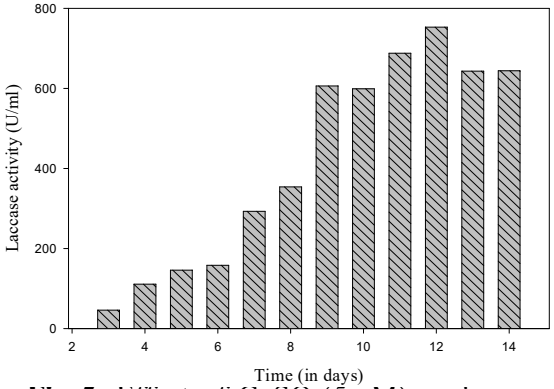



**Fig 5.** Effect of $CuSO_4$(5mM) on laccase production by *Ganoderma*sp. Cultures

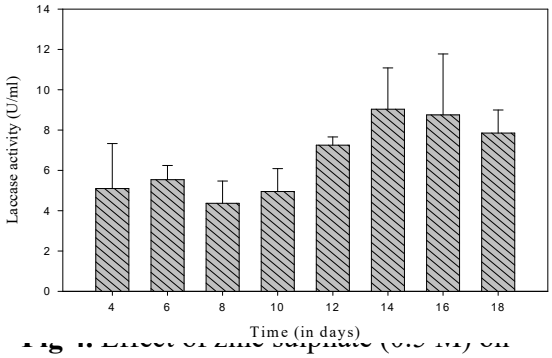



**Fig 4.** Effect of Zinc sulphate (0.5 M) on laccase production by *Ganoderma*sp. Cultures

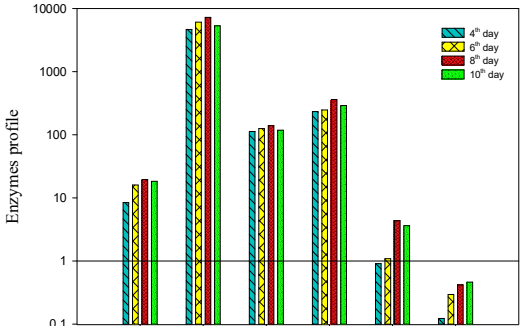


**Fig 6.** Production profile of various e ıder c

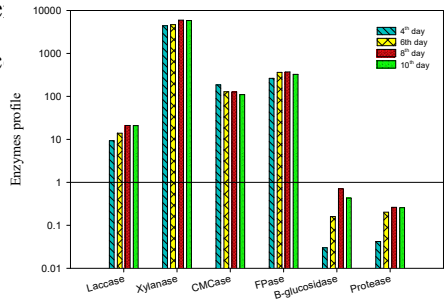



**Fig 7.** Production profile of various enzymes on eucalyptus and pomegranate waste under co-cultivation condition

**Laccase Production under SSF conditionusing mixed substrate**

Laccase activity was maximum (24 U/g) when we used pomegranate peel as a substrate along with wheat bran in the ratio of 1:1. While xylanase activity was maximum when we used kitchen waste (7239 IU/g), CMCase and FPase activity was high with pomegranate (208 IU/g and 419 IU/g), respectively; while β-glucosidase activity was maximum with kitchen waste (4.34 IU/g) on 8th day (Fig. 6).

In comparison with other enzymes protease activity was found to be low in case of kitchen waste, while with eucalyptus and pomegranate, protease activity was very similar to β-glucosidase activity (Fig. 7).

**Zymogram (Native PAGE) analysis of laccase isozymes**

Multiple isoform of laccases were observed when grown on different solid substrate under SSF condition (Fig. 8). The zymogram analysis showed the presence of different isozymes with different substrates

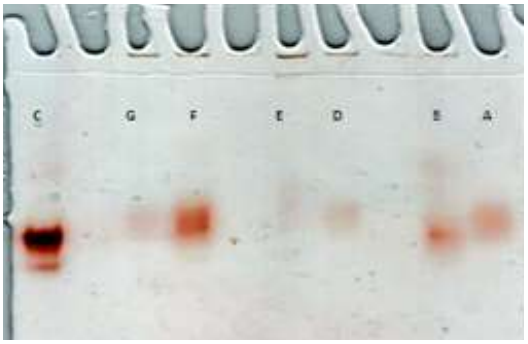



**Fig 8.**Zymogram of Native PAGE. (A) Kitchen waste, (B) Kitchen waste, (C) Control, (D) Pomegranate peel, (E) Pomegranate peel waste, (F) Eucalyptus waste.

**Discussion**

The biodegradation of lignin holds a significant position in the global carbon cycle since it constitutes the second largest sink for fixed carbon after cellulose. White rot fungi are the only organisms with a demonstrated capacity to mineralize lignin; consequently, the enzyme mechanisms they employ for lignin breakdown have attracted considerable research interest (Kirk and Farrel, 1987; Eggert et al. 1996). Basidiomycetous fungus was identified as

*Ganoderma*sp. based on morphological, cultural and microscopic observations (Fig 4.1 and 4.2). Plate assay confirmed the isolate to be laccase positive as it oxidizes guaiacol to give reddish brown color (Fig 4.3). The maximum day of enzyme production was found to be 4th day with 8.30U/ml of activity (Fig 4.4)

Laccase production under submerged cultivation conditions has been reported extensively but reports on solid-state fermentation (SSF) are not many (Vasdev and Kuhad, 1994; Palmieri et al. 2003; Sharma et al. 2005). Earlier, solid-state fermentation has emerging as a major thrust area for producing enzymes due to considerable economic potentials offered in terms of high volumetric productivity, low capital costs, reduced energy requirement and simple fermentation media (Tellez et al. 2008).

Laccases are generally produced in low concentrations by laccase-producing fungi but higher concentrations were obtained with the addition of various supplements such as xenobiotic compound to media (Sharma et. al. 2005). Copper is the most frequently applied substance to enhance laccase production in fungi (Palmieri et. al. 2000; Jain et. al. 2020). It is part of the active center of laccases, and is thus crucial for the synthesis of a catalytically active laccase protein (Baldrian et al., 2003). Several reports on laccase induction have been published in past; *G. lucidum* 447 with 1mM, 3mM $CuSO_4$ gave 578.2 U/ml, respectively (Songulashvili et al. 2011), *P.ostreatus* with 1mM $CuSO_4$ reported laccase activity of 570 U/L (Kumar et al. 2011). The effect of metal ions was studied using different metal ions such as $ZnSO_4$, $FeSO_4$, and $CuSO_4$ at predefined concentration of 5mM to induce the production of laccase. Copper sulphate was found to be most potent inducer for laccase production i.e. 753 U/ml (90.71-fold) on 12th day. Whereas, other metal ion i.e. $ZnSO_4$ and $FeSO_4$ have no significant effect on laccase production

When co-cultivation was done under SSF condition, laccase activity was maximum when we used pomegranate peel as a substrate along with wheat bran in the ratio of 1:1. In comparison with other enzymes protease activity is low in case of kitchen waste, while with eucalyptus and pomegranate protease activity is very similar with β-glucosidase activity.

Zymogram of Native- PAGE analysis showed the presence of different isozymes with different substrates.

**Conclusion**

The interest in utilizing ligninolytic enzymes for biotechnological applications has increased rapidly since the discovery of these enzymes in white-rot fungi.

Laccases are the versatile enzymes which catalyze oxidation reactions coupled to four-electron reduction of molecular oxygen to water. They are multi-copper enzymes which are widely distributed in higher plants and fungi. They are capable of degrading lignin, and are present abundantly in many white-rot fungi. SSF is a very promising cultivation technique for the production of industrially-relevant enzymes such as laccase. Laccases are ancient enzymes with a promising future. This class of enzymes still remains of great relevance both as a model for structure-function relationships and as a green tool in many processes of the biotechnology industries in the near future.

**Acknowledgement**

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