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STUDY OF ISOLATION OF LACCASE PRODUCING FUNGI AND ITS APPLICATION IN PLANT GROWTH PROMOTION

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Abstract

The rapid accumulation of lignocellulosic agricultural wastes poses a serious environmental challenge due to its persistence during biodegradation and the release of pollutants during improper disposal. Lignin, a major structural component of agro-waste, is particularly resistant to conventional physical, chemical and biological methods of waste degradation. This study aimed to isolate and characterize an efficient laccase-producing fungal strain and evaluate its potential application in lignocellulosic waste bioconversion. Among the 65 isolates screened from 6 samples, only 4 isolates demonstrated laccase activity and *Lividopora benetosta* (NCBI accession No. OR262175) was identified as the most potential strain. Optimum production of laccase (1.23 U/mL) by *L. benetosta* was observed in potato dextrose broth (pH 7) containing 2% glucose,on incubation at room temperature under shaking conditions and absence of inducer (guaiacol).The practical applicability of the crude enzyme was assessed based on cocopeat treatment. Effective lignin degradation was observed on laccase application, and demonstratedfaster seed germination (within 1–2 days), increased root development, and reduced wilting compared to untreated controls in both monocot (*Triticum aestivum*) and dicot (*Vigna radiata*) plants. The *T. aestivum* seedlings showed approximately 25% larger shoot length, while *V. radiata* plants over 50% improvements in both shoot and root growth. These results demonstrate the potential of *L. benetosta*derived laccase as an eco-friendly and sustainable tool for agrowaste management and soil improvement.

Keywords: *eco-friendly, laccase, lignin, lignocellulosic agricultural wastes, Lividopora benetosta, sustainable.*

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1. INTRODUCTION

Agricultural wastes comprises of residual by-products generated during farming activities. It includes crop residues such as straw, stalks, leaves, coconut husks, and sugarcane bagasse. A major factor contributing to the recalcitrance of agricultural wastes is its lignocellulosic composition. It is one of the most abundant renewable resources on earth consisting primarily of cellulose (40–50%), hemicellulose (25–30%), and lignin (15–20%) [1]. Among these components, lignin presents the most resistant barrier to degradation methods due to its highly heterogeneous and complex aromatic structure. Lignin is composed of guaiacyl, syringyl and p-hydroxyphenyl units interconnected through strong ether and carbon–carbon bonds, which forms a complex and rigid matrix within plant cell walls and prevents enzymatic access to cellulose and hemicellulose structures [2]. The improper disposal and continuous accumulation of these agricultural wastes poses serious environmental challenges such as air and water pollution, greenhouse gas emissions, soil degradation, and eutrophication of aquatic ecosystems [3]. Practices like open burning is also common which releases carbon dioxide, methane, and particulate matter in air, contributing to air pollution [3,4]. Another common practice is disposing of these agricultural wastes along with municipal wastes, which are often dumped in landfills, where they decompose anaerobically leading to methane generation [3,5]. Consequently, the sustainable management of agricultural wastes, especially the lignocellulosic content has become an urgent global concern [3].

Conventional physicochemical methods used for lignin removal include steam explosion, alkaline hydrolysis and chlorine-based treatments [6]. Though these methods are effective, they are energy-intensive and have detrimental environmental effects due to generation of toxic by-products, volatile organic compounds and hazardous effluents [7]. Consequently

the research focus in management of agricultural wastes has shifted from physical and chemical methods towards environmentally sustainable biological approaches for bioremediation of lignin [8]. The biological degradation of lignin is primarily mediated by ligninolytic enzymes, including laccases, manganese peroxidases, and lignin peroxidases, which catalyze the oxidative depolymerization of lignin [9,10]. These enzymes are produced by a variety of microorganisms, including fungi, bacteria, and actinomycetes. Among these, white-rot fungi such as *Trametes versicolor*, *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, and *Ganoderma lucidum*are particularly recognized for their lignin degrading ability [11].

Laccases are multi-copper oxidase enzymes that play a central role in lignin degradation by catalyzing the oxidation of phenolic and non-phenolic lignin compounds while reducing molecular oxygen to water. Structurally, laccases contain four copper ions organized into Type 1, Type 2 and Type 3 copper centers, which facilitate efficient electron transfer during substrate oxidation [12]. This unique catalytic mechanism enables laccases to act on a wide range of substrates, making them highly versatile enzymes with applications in bioremediation, wastewater treatment, pulp and paper processing, textiles as well as biofuel production [12,13]. Fungal laccases are particularly preferred over bacterial and plant laccases due to their higher catalytic efficiency, broader substrate specificity and greater stability under varying pH and temperature conditions [14,15]. Advances in biotechnology and protein engineering have further enhanced laccase activity, allowing their use under harsh industrial conditions and in combination with other ligninolytic enzymes to improve lignin degradation efficiency [16].

Despite their extensive industrial relevance, large-scale application of laccases is constrained by challenges such aslimited enzyme yields by microorganisms and high production costs [16]. For this reason, research on isolation of novel and high yielding enzyme producing microbial strains,characterization of enzymes and application based studies in sustainable management of agricultural wastes is the primary focus in microbiological studies. In this context, the present study aimed to contribute to waste management practices and explore the potential applications of fungal laccases in plant growth promotion. The objectives of the study were to isolate and characterize efficient laccase-producing fungi from natural sources, evaluate their laccase activity, and assess the applicability of crude laccase in the treatment of cocopeat (lignocellulosic substrate).

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All chemicals and reagents used in this study were of analytical grade and procured from certified commercial suppliers. Carbohydrates including dextrose, sucrose, maltose, mannitol, xylose, cellobiose, galactose, mannose, starch and lactose were obtained from LOBA Chemicals (India). Agar and guaiacol, required for culture media preparation and enzymatic assays respectively, were sourced from HiMedia Laboratories Pvt. Ltd. (India). Sodium acetate and glacial acetic acid, used for buffer preparation and pH adjustments, were also procured from LOBA Chemicals. All reagents were handled and stored according to manufacturer recommendations to ensure consistency and reliability throughout experimental procedures.

2.2 Sample collection

Laccase producing fungi were isolated from soil samples, wood swabs, and other organic materials collected from various locations within the Wilson college premises and nearby residential areas in Mumbai. Soil samples were transferred into clean plastic bags, whereas decaying tree bark samples were collected using sterile cotton swabs to minimize contamination. To enhance the diversity of fungal isolates, additional substrates including spoiled fruits, molded bread, and decomposed vegetables were also sampled. Additionally, to capture airborne fungal spores, sterile 9 cm potato dextrose agar (PDA) plates were exposed to ambient air.

2.3 Isolation and maintenance of fungal isolates

The soil and wood samples were suspended in sterile saline and vortexed for 2–3 mins to effectively dislodge fungal spores from the substrate surfaces. The resulting suspensions were inoculated onto PDA plates and incubated at room temperature (RT; ~25–30°C) for 3–4 days to facilitate fungal growth. Emerging fungal colonies were carefully transferred and purified through repeated sub-culturing onto fresh PDA plates until distinct, pure cultures were obtained. The pure isolates were maintained on PDA slants at 4°C and sub-cultured monthly to preserve viability.

2.4 Screening of Laccase producers

2.4.1 Plate Assay Method

Primary screening of fungal isolates for laccase production was performed using a qualitative plate assay described byThrimothi [17]. Purified fungal isolates were spot-inoculated onto PDA plates supplemented with 4 mM guaiacol and 4 mM tannic acid as indicator substrates. The inoculated plates were incubated at RT and monitored at 24 h intervals for up to two weeks. The development of a reddish-brown halo around the fungal colonies was recorded as a positive indication of laccase activity, resulting from the oxidative polymerization of guaiacol and tannic acid.

2.4.2 Bavendam Test

Secondary screening for laccase production was carried out by transferring 4–5 fungal agar plugs (approximately 5 mm in diameter, cut using a cork borer) aseptically into test tubes containing 10 mL of sterile potato dextrose broth (PDB). The broth was supplemented with 4 mM guaiacol, which produces characteristic brownish-red coloration upon oxidation. The inoculated tubes were incubated at RT under static conditions to facilitate fungal growth and enzyme

secretion. Throughout the incubation period, the cultures were routinely observed for the development of reddish-brown coloration in the medium, which served as a qualitative indicator of laccase activity [18].

2.5 Identification of potential isolate

The most potential laccase producing isolate was identified based on morphological and molecular methods.

2.5.1 Slide culture technique

A clean, grease-free glass slide was positioned on a sterile U-tube support within a 9 cm Petri plate lined with a moistened cotton sheet to maintain humidity. The entire setup was autoclaved at 121°C and 15 psi to ensure sterility. After cooling, a small drop of molten PDA was placed at each end of the slide, allowed to solidify, and gently divided into two sections, leaving a narrow gap between them. Fungal inoculum was transferred onto the agar drops using a cotton swab, and each inoculated area was covered with a sterile coverslip. The prepared slide culture was incubated at RT for approximately two weeks. Slides were examined at 24 h intervals to observe hyphal development, reproductive structures, and spore formation [19].

2.5.2 Lactophenol cotton blue staining

For microscopic examination of fungal structures, culture smears were prepared and stained with 0.1% lactophenol cotton blue. The stained smears were observed under a light microscope at 40× magnification to visualize key morphological features, including hyphae, spore morphology, and the presence of conidiophores [20].

2.5.3 Molecular identification using ITS sequencing

For molecular identification, freshly isolated fungal cultures were submitted to Saffron Lite Laboratories (Gujarat, India) for Internal Transcribed Spacer (ITS) sequencing. The ITS region of the ribosomal DNA is a highly variable genetic marker widely used for fungal identification. This region was amplified and analyzed to accurately identify the most promising laccase-producing fungal isolate.

2.6 Quantitative estimation of enzyme production

To prepare the fungal inoculum, actively growing mycelial cultures aged four days on PDA plates were used. Approximately 5 mm agar were excised from the plates using sterile cork borers and aseptically transferred into 150 mL Erlenmeyer flasks containing 50 mL of sterile PDB. Inoculated flasks, along with uninoculated abiotic controls, were incubated on a rotary shaker at RT for 7 days to promote mycelial growth and laccase enzyme production. The laccase activity was quantified using the guaiacol oxidation assay, with guaiacol serving as the chromogenic substrate. The reaction mixture for the test sample consisted of 1 mL of crude enzyme extract, 3 mL of sodium acetate buffer (pH 5.5), and 1 mL of 2 mM guaiacol. In the control tube, the enzyme extract was replaced by sodium acetate buffer. All tubes were incubated at RT for 15 mins, and the absorbancewas measured using a UV–Vis spectrophotometer at 467.6 nm. Enzyme activity was expressed in International Units (IU), where one IU corresponded to the amount of enzyme required to oxidize 1 μmol of guaiacol per minute [21]. Laccase activity (U/mL) was calculated using the Eq. 1:

$$\text{Laccase activity (U/mL)} = \frac{\text{A x V}}{\text{t x e x v}} \ldots \text{Eq. 1}$$

Where 'A' is the absorbance at 467.6 nm, 'V' is the Total reaction volume (ml), 'v' is the enzyme volume (ml), 't' is the Incubation time (min) and 'e' is the Extinction coefficient (0.6740 mcgM/cm).

2.7 Optimization of various parameters for enhanced laccase production

The physicochemical parameters optimized for laccase production by the most promising isolate were oxygen availability (static or shaker conditions), presence of 1-5% w/v carbon sources (galactose, xylose, mannose cellobiose, sucrose, starch, maltose, mannitol, and lactose), pH (3, 4, 5, 6, 7, 8, 9, and 10), temperature (RT, 35°C, 45°C, and 55°C) and presence of inducer (2 mM guaiacol) [22-25]. In each set up, 50 ml of sterile PDB medium was inoculated with the laccase producing isolate and incubated for 7 days. Aliquots of 5 ml were collected from the flasks at 24 h intervals, and laccase enzyme activity was quantified using the guaiacol oxidation method described above [21].

In addition to the physicochemical parameters, potato based and rice based media formulations were tested for enzyme production. In one set, 20 g of potato chunks/ rice were boiled in distilled water for 30 mins, after which the broth was filtered and supplemented with 2g of dextrose. In another variation of this media, potato chunks/ rice were simply rinsed and immersed in distilled water instead of boiling, and then supplemented with 2g of dextrose. Additionally, these media were tested for laccase production with and without dextrose.After media preparation, fungal agar plugs were inoculated into each flask and incubated at RT for one week. Laccase activity in different media was measured daily using the guaiacol oxidation assay.

2.8 Purification of laccase Enzyme

Partial purification of crude laccase was carried out using ammonium sulfate precipitation method26. Fourteen fungal agar plugs were inoculated in 100 mL PDB and incubated for 96 h at RT under continuous shaking conditions. Following incubation, the culture was filtered through Whatman filter paper (pore size 10 μm), and the clear filtrate was collected in a sterile 250 mL beaker. Ammonium sulfate was gradually added to the filtrate with constant stirring on a magnetic stirrer, following standard ammonium sulfate saturation tables, until complete dissolution indicated the desired saturation level. The saturated mixture was transferred to a 150 mL flask and refrigerated for 48 h to promote protein precipitation.After refrigeration, the precipitated proteins were collected by centrifugation at 5500 rpm for 30 mins. The supernatant was discarded, and the resulting pellet was

carefully re-suspended in 3 mL of 1 M sodium acetate buffer (pH 5). The re-suspended protein solution was then transferred into a pre-activated dialysis bag (pore size 10 µm) and dialyzed against 0.01 M sodium acetate buffer for 24 h, with the external buffer replaced every 6 h to ensure efficient removal of excess salts. Upon completion of dialysis, the purified enzyme extract was collected in sterile tubes and subsequently analyzed for laccase activity using the guaiacol oxidation assay.

2.9 Determination of Molecular Weight of enzyme

The molecular weight of the partially purified laccase enzyme was determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme sample was loaded onto the gel alongside a standard protein molecular weight marker to determine the approximate molecular weight of the laccase protein [27]. Following electrophoresis, the gel was stained and examined for distinct protein bands corresponding to the partially purified enzyme.

2.10 Application of laccase for cocopeat treatment

2.10.1 Cocopeat treatment

To evaluate the effect of crude laccase on cocopeat, 200 mL of PDB was inoculated with fungal agar plugs and incubated at RT for four days to allow enzyme production. Following incubation, the fungal biomass was removed by filtration through Whatman filter paper, and the resulting crude enzyme filtrate was collected for subsequent treatment. A total of 50 mL of crude laccase extract was applied to 500 g of commercial cocopeat on alternate days over a three-day period. After each application, the mixture was thoroughly homogenized to ensure uniform enzyme distribution. The treated cocopeat was then allowed to stand undisturbed for ten days to facilitate enzymatic action. Following this incubation period, the laccase-treated cocopeat was used for planting seeds.

2.10.2 Pot assay

A pot assay was conducted to evaluate the effect of laccase-treated cocopeat on seed germination and early plant growth. Two experimental systems were established. In the first system, 100 g of laccase-treated cocopeat was placed into plastic pots, each containing five drainage holes to ensure adequate aeration and water removal. Control pots were prepared using an equivalent amount of untreated cocopeat. Seeds of wheat (*Triticum aestivum*) andmoong (*Vigna radiata*), representing monocotyledonous and dicotyledonous plants respectively, were selected for the study. All seeds were soaked overnight in sterile distilled water for uniform hydration before sowing. Five seeds were planted per pot at an approximate depth of 2 cm. The experiment was performed in duplicate, and pots were watered daily with sterile distilled water for 15 days.

In the second system, the same planting procedure was followed; however, instead of using laccase-treated cocopeat, untreated cocopeat was used in all test pots. In this setup, crude laccase filtrate was applied directly during watering at regular intervals throughout the 15day experimental period, while control pots were moistened with tap water. Across both systems, germination rates, seedling height, and root development were carefully monitored to assess the influence of laccase treatment on plant growth.

3. RESULTS AND DISCUSSIONS

3.1 Isolation of laccase producing fungi

Laccase producing fungi are widely distributed in soil and decomposing woody substrates28. Hence, soil, wood and compost samples were screened in this study for isolation of laccase producing fungi. Additionally, air sampling was done to capture spores of laccase producers; however, none of the 20 isolates obtained on PDA plates produced laccase. Altogether,a total of 65 distinct fungal isolates (Table 1) were obtained in this study. Among these, only four isolates (S3, S4, S8, S19) exhibited laccase activity. This relatively low percentage of laccase producers aligns with findings from previous studies. For example, Vantamuri et al.29 reported prevalence of 5–6% laccase producing isolates in agro-waste samples screened in their study. The limited percentage of laccase producers could be due to species-specific regulation, genetic variation, or environmental conditions, which restrict the expression of laccase to a small subset of fungal strains30. Additionally, nutrient availability in the growth medium may have a possible influence on laccase production [28].

Guaiacol supplementation in media allows for easy identification of laccase activity, which is manifested as reddish-brown halos around the fungal colonies in the plate assays.On the other hand, thebroth assay allows more rapid detection of enzyme activity. Among the laccase positive isolates, isolate S8 exhibited the most rapid response of color change to brownish red, within 3 h, which continued to intensify over 24 h. In contrast, S3 and S4 required 24h, and S19 required 48h to show comparable activity. The absence of agar in liquid media allows for greater enzyme mobility, leading to faster interactions with the substrate and quicker color changes.Consequently, broth assays are preferred over plate assays for detection of enzyme activity31. In this study, guaiacol oxidation assay confirmed a progressive rise in enzyme activity of isolate 8, with the highest levels recorded at 72 h of incubation, reaching a peak value of approximately 1.235 U/mL (Fig. 1). A gradual decline in activity was noted beyond day 3, suggesting that enzyme production and activity are optimal during the early and active stages of fungal growth.

Table 1: Sources and distribution of isolates collected from various environmental samples

Sample Source		Isolates
Garden soil	Wilson College	S1, S2, **S3, S4**, S5, S6
	Residential area	S27, S28, S29, N1, N2, P1, P2, P3
	Potted plants	S18, **S19**, S20, S21, S22, S23, S24, S25, S26
Wood	-	S7, **S8**, S9, S10, S11, S12,

		S13, S14, S15, S16, S17
Air flora	-	M1, M2, M3, M4, M5, M6, M7, M8, M9, M10, M11, M12, M13, M14, M15, M16, M17, M18, 19, M20
Compost	-	C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11

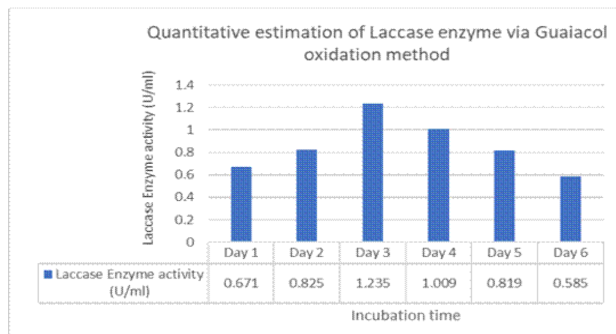

Fig. 1: Quantitative estimation of laccase enzyme using Guaiacol oxidation assay method

3.2 Identification of potential isolate

Based on qualitative tests, S8 isolate was recognized as the most potential laccase producer. Hence, it was characterized morphologically and identified based on molecular methods. The lactophenol cotton blue staining of the isolate revealed fungal hyphal structures, but no spore-producing bodies or vesicles were observed under microscopic examination (Fig. 2a, 2b). Since sporulation is dependent on specific environmental conditions, spores are not commonly detected with lactophenol cotton blue staining method20. Hence, slide culture technique was employed to observe the fungal morphology more comprehensively over an 11-day period. Mycelial growth was first observed on day 3 (Fig. 2c), and by day 11, small diamond-shaped structures (likely representing spores) along with hyphal fragmentation were observed (Fig. 2d). Typically, fungi sporulate through hyphal fragmentation when conditions are suboptimal for fruiting body formation such as in decaying wood [32].

The BLAST analysis showed a highly significant match with *Lividopora benetosta* voucher Otto Miettinen 18035 (H) (sequence ID: OR262175.1), based on the small subunit ribosomal RNA gene (Fig. 3). The alignment showed a perfect identity score of 595/595 (100%) with no gaps, and an E-value of 0.0, indicating a highly reliable match. *L. benetosta*is not typically associated with laccase production; hence exploring such novel strains may prove to be particularly significant for industrial applications. Most studies on laccase production have focused on species such as *Pleurotus ostreatus*, *Ganoderma lucidum* and *Trametes versicolor* [33].The phylogenetic tree (Fig. 4) illustrated the evolutionary relationships between *L. benetosta*and Ganoderma sp., but this relationship was weakly supported by a low bootstrap value of 37%. Instead, it showed closer evolutionary relationships with *Hygrocybe intermedia* and *Rigidoporus vinctus* with higher bootstrap values of 68%.

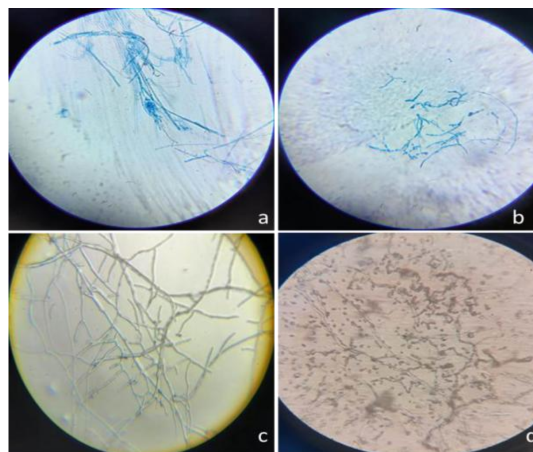

Fig. 2: Microscopic examination of *Lividopora benetosta* under 40x magnification
(a) and (b) represents fungal hyphal structures observed with lactophenol cotton blue staining; (c) and (d) represents fungal mycelial growth observed with slide culture on day 3 and day 11.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Lividopora benetosta voucher Otto Miettinen 18035 (H) small subunit ribosomal RNA gene	Lividopora benetosta	1074	1233	100%	0	100.00%	1550	OR262175.1
Ganoderma sp. isolate Tsugae internal transcribed spacer 1, partial sequence	Ganoderma sp.	1187	1375	100%	0	98.83%	688	OQ284066.1
Rigidoporus vinctus isolate MEBP0032 small subunit ribosomal RNA gene	Rigidoporus vinctus	1088	1247	100%	0	99.35%	660	MT597859.1
Cordyceps militaris isolate SR4009 small subunit ribosomal RNA gene	Cordyceps militaris	1073	1232	100%	0	99.83%	656	PP830047.1
Lividopora benetosta voucher Nils Hallenberg 15301 (O) internal transcribed spacer 1	Lividopora benetosta	1069	1178	96%	0	99.83%	851	OR262161.1
Basidiomycota sp. voucher biocode09-387 internal transcribed spacer 1	Basidiomycota sp.	1069	1214	99%	0	99.83%	1192	MZ996950.1
Fungal endophyte isolate 2307 internal transcribed spacer 1, partial sequence	fungal endophyte	1069	1228	100%	0	99.83%	685	KR015215.1
Hygrocybe intermedia isolate BMS15F small subunit ribosomal RNA gene	Hygrocybe intermedia	1069	1228	100%	0	99.83%	758	OR099729.1
Basidiomycota sp. voucher biocode09-393 internal transcribed spacer 1	Basidiomycota sp.	1069	1227	100%	0	99.83%	1215	MZ996951.1
Lividopora benetosta isolate NZFS163A(=FRI163/1) internal transcribed spacer 1	Lividopora benetosta	1069	1227	100%	0	99.83%	715	PP848982.1

Fig. 3: BLAST analysis report

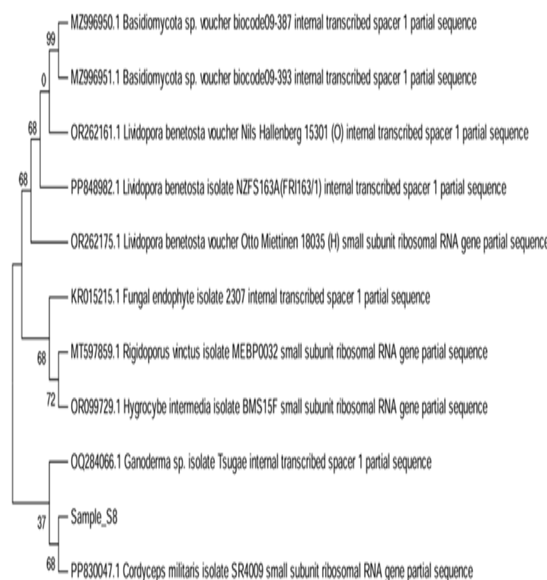

Fig. 4: Phylogenetic tree of isolate 8

3.3 Optimization of environmental and nutritional parameters

To optimize laccase production by *L. benetosta*, various growth parameters were optimized. Based on tested parameters, the optimal conditions were identified as PDB medium with 2% glucose, pH 7, incubation at RT under shaking conditions, and absence of guaiacol (Fig. 5). The results suggest that oxygen availability plays a critical role in enhancing enzyme activity. Besides glucose, carbon sources such as mannitol, cellobiose, and starch supported moderate enzyme production, whereas xylose and lactose resulted in minimal activity. Temperature optimization revealed that ambient temperatures (25–30°C) supported maximum fungal growth and laccase production. Although fungal growth was limited at 45°C and 55°C, low levels of laccase activity were still detected, indicating a degree of thermal tolerance. The addition of guaiacol as an inducer did not affect laccase activity indicating that *L. benetosta* does not require chemical induction for effective laccase synthesis.

Laccase production was also strongly influenced by pH, with maximum activity observed between pH 6-7. Buffer system and pH have profound effects on laccase activity and stability, and most fungal laccases showacidic optima in the pH range between 3 and 6, depending on substrate and organism [15]. For instance, *Pleurotus ostreatus* NRC620 showed optimum growth at 28°C and optimum laccase activity at pH 3.0, and 70°C34. Similarly, *Trichoderma harzianum* AUMC14897 isolated from *Opuntia ficus-indica* showed optimum activity of pure laccase at 50°C and pH 4.5, with thermal stability up to 50°C35.Similar to *L. benetosta* which produced 1.235 U/ml laccase in this study, white rot fungi such as *Pleurotus ostreatus* NRC 620, *Pleurotus sajor-caju*and *Trametes versicolor*produced 1.390 U/ml, 1.450 U/gand 1.588 U/ml laccase over a period of 10-20 days [34,36,37]. *Trametes hirsuta* has been reported to reach extracellular laccase activities of up to 11 U/mL in bioreactors [38].

3.4 Effect of various media formulations on enzyme activity

The effect of media formulations on enzyme activity is illustrated in Fig. 6. Among the tested formulations, media with boiled potato and dextrose yielded the highest laccase activity (1.045 U/ml), followed by media with boiled rice and dextrose (0.991 U/ml). Since boiling enhances nutrient availability, it supports better growth and higher laccase production. Even in absence of dextrose, the media formulated with potato, both rinsed and boiled, showed strong enzyme activity of 0.977 U/ml and 0.953 U/ml, respectively. Comparatively, rice-based media exhibited lower activity, though boiling still improved enzyme yields. A similar study on use of alternate economic media formulation showed suitability of wheat straw for laccase production by *Pleurotus sajor-caju*. This strain produced 1450 U/g laccase in media (pH 6.0) containing 1% fructose, 0.5% peptone and 7.5% inoculum size on incubation at 26°C and72.5% moisture content [36]. In another study, *P. ostreatus* and *Flammulina velutipes* strains showed better laccase activity in presence of cottonseed hull and stalk of straw, respectively, as compared to corncob [39]. These findings suggest that suitable agro-wastes can be useful for economic production of laccase in the industries.

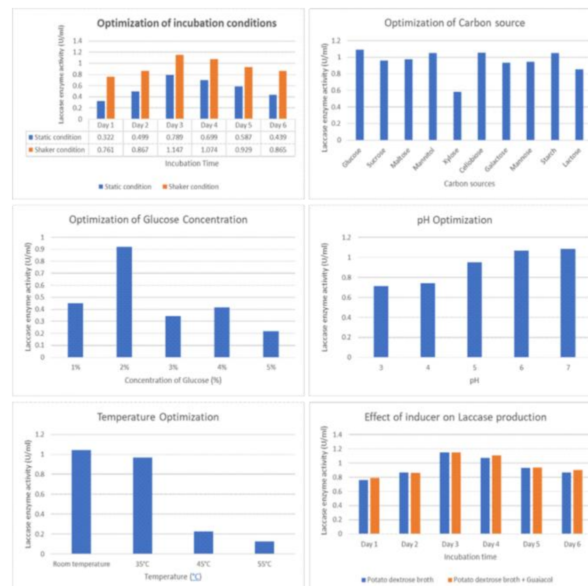

Fig. 5: Optimization of growth parameters on laccase production by *L. benetosta*

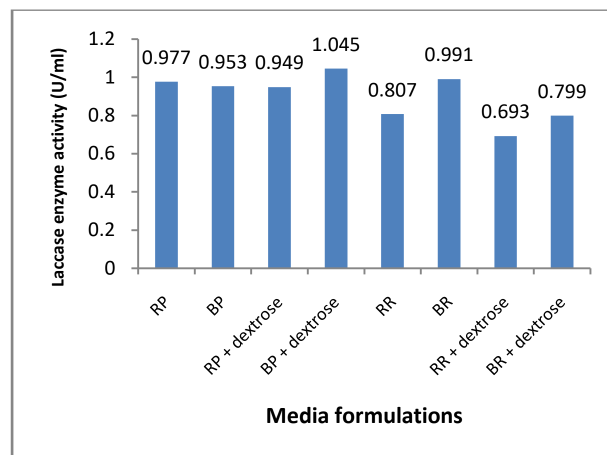

Fig. 6: Effect of media formulations on laccase production by *L. benetosta*
(RP: media with rinsed potato; BP: media with boiled potato; RR: media with rinsed rice; BR: media with boiled rice

3.5 Purification of Laccase enzyme

In this study, a decrease in enzyme activity was observed post-purification. In the crude extract, laccase activity increased steadily from Day 1 (0.661 U/mL) to reach a maximum on Day 3 (1.285 U/mL), followed by a gradual decline on day 4-6. However, for purified laccase, the activity increased from 0.558 U/mL on Day 1 to 0.870 U/mL on Day 3. The reduced activity observed after purification suggests partial loss

of enzyme activity during the purification process, possibly due to protein denaturation or removal of stabilizing factors present in the crude extract [40]. It may also be due to the heat generated during the mixing of ammonium sulfate with the aid of a magnetic stirrer or centrifugation process41. Although ammonium sulfate precipitation is a widely used initial step for enzyme purification, it does not always enhance enzyme activity. For example, esterase from *Sorghum bicolor*also exhibited reduced activity at higher ammonium sulfate saturation levels (above 60%)42. In one study on purification of laccase from *Curvularia lunata* MY3, ammonium sulfate precipitation was followed by Sephacryl S-200 and DEAE-Sepharose chromatography to increase specific activity and purification fold of enzyme43. Since this study was designed keeping in mind the economic and environmental perspectives, the subsequent purification attempts were not made. Instead, application of crude enzyme was studied on lignin degradation and plant growth promotion.

3.6 Application of Laccase in Lignin Degradation

The observations of this study clearly demonstrated better plant growth in experimental system 1 which used pretreated cocopeat, as compared to experimental system 2 where untreated cocopeat in pots were watered with crude laccase enzyme during plant growth. The efficacy of laccase treated cocopeat in supporting plant growth is represented in Fig. 7. In this study, both wheat (monocot) and moong (dicot) seeds sown in pretreated cocopeat showed faster germination within 1–2 days, with 100% germination rate. In contrast, the seeds in control set up germinated in up to 4 days, and showed ~70% germination rate (Table 2).Overall, the treated plants exhibited enhanced shoot and root development, reduced wilting, and overall improved vigor relative to controls. The number of leaves was also higher in the test groups compared to the controls, suggesting improved vegetative development in the treated plants (Fig. 7).

The shoot length was significantly enhanced in both crops grown in pretreated cocopeat. Moong plants exhibited a mean shoot length of 13.45 cm compared to 7.15 cm observed in controls ($t = -10.42$, $p = 0.0304$), while wheat plants showed a mean shoot length of 16.02 cm compared to 10.85 cm in controls ($t = -19.38$, $p = 0.0161$). Similarly, the roots were significantly more developed in both crops grown in pretreated cocopeat. The roots of moong plant were approximately 19.2 cm compared to 8.6 cm in controls ($t = -17.67$, $p = 0.0179$), while wheat plants showed a mean root length of 28.2 cm compared to 17.5 cm in controls ($t = -35.67$, $p = 0.0089$). These results indicate a statistically significant positive effect of pretreated cocopeat on shoot and root elongation.

In the experimental system 2, regularly watered with crude laccase enzyme, longer mean shoot lengths were observed compared to controls; however, the differences were statistically insignificant for wheat ($t = -5.88$; $p = 0.0536$) as well as moong ($t = -2.03$; $p = 0.145$). On the other hand, the root length of both plants showed a statistically significant improvement. Precisely, roots of wheat were approximately 21.5 cm in test pots and 15.0 cm in control pots ($t = -13$; $p = 0.0244$) and roots of moong were 17.2 cm in test pots and 8.1 cm in control pots ($t = -91$; $p = 0.0035$). This suggests that while direct enzyme application may transiently affect the rhizosphere possibly due to enhanced localized lignin degradation and root penetration, the effects are less pronounced and less sustained than those achieved through substrate pretreatment. Most probably, this may be due to enzyme instability and dilution effects during irrigation that may reduce the overall impact of laccase when applied post-planting [44,45].

In summary, the present study demonstrates that pretreated cocopeat outperforms systems where crude laccase was applied only through irrigation.

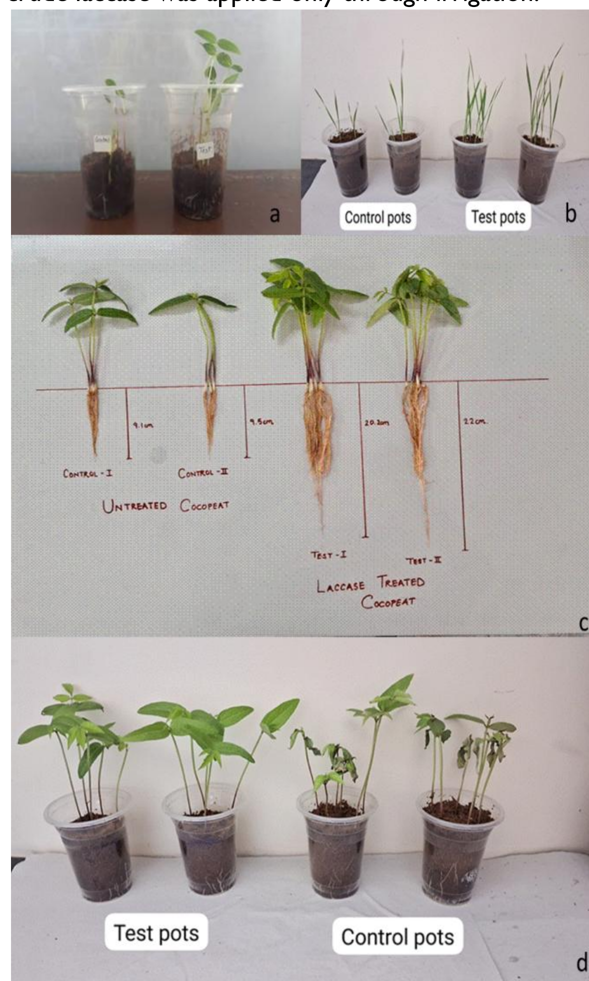

Fig. 7: Efficacy of laccase treated cocopeat on plant growth

The figure shows comparison of growth in control and test pots with (a) moong plant and (b) wheat plant; (c) comparison of root development in control and test plant samples; (d) plants grown in untreated cocopeat showing wilting after 11 days of growth.

Table 2: Sources and distribution of isolates collected from various environmental samples

Growth parameters observed in Moong plantlets				
	Fortified experiment		Enzyme watering experiment	
PARAMETERS	CONTROL	TEST	CONTROL	TEST
No of seeds germinated	3.5	5	3.5	5
Shoot length	7.1	13.4	10.2	13.8
Number of leaves	13.5	21.5	13.5	17.5
Length of root	8.6	19.2	8.1	17.2
Growth parameters observed in Wheat plantlets				
	Fortified experiment		Enzyme watering experiment	
PARAMETERS	CONTROL	TEST	CONTROL	TEST
No of seeds germinated	4.5	5.5	3.5	5
Shoot length	10.8	16.	6.5	8.7
Number of leaves	5.5	11	5	8.5
Length of root	17.5	28.2	15	21.5

Rapid and uniform seed germination observed in the laccase-treated cocopeat suggests that enzymatic lignin degradation improved substrate physicochemical properties at an early stage. Lignin is known to impart hydrophobicity and structural rigidity to plant residues, limiting water absorption and nutrient accessibility46. Laccase pretreatment of lignocellulosic residues has been shown to significantly reduce lignin and phenolic content, altering the physicochemical structure of the substrate and facilitating enhanced microbial accessibility and saccharification [47,48]. These structural changes are hypothesized to improve substrate suitability for microbial activity and nutrient cycling, which can in turn support plant growth when residues are applied as soil amendments.

4. CONCLUSION

The findings of this study highlight the significant potential of laccase-producing *L. benetosta*for eco-friendly agro-waste treatment and enhancement of plant growth. Laccase-mediated lignin degradation in cocopeat likely reduced substrate recalcitrance, enhanced microbial activity, and improved nutrient mineralization and moisture retention. These combined effects created a more favorable growth environment for plants, resulting in improved germination and vegetative development. Overall, the results demonstrate that enzymatic pretreatment of lignin rich agrowaste substrates such as cocopeat using laccase represents a sustainable and effective strategy for improving substrate quality, nutrient availability, and water-holding capacity. Collectively, this approach allows improved crop establishment and development while simultaneously offering an environmentally responsible pathway for agro-waste valorization.

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7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

8. INFORMED CONSENT

Not applicable

9. ETHICAL STATEMENT

Not applicable

10. AUTHOR CONTRIBUTION

Radhika D. Birmole - Concept, Design, Analysis, Writing. Sufiyan Jalgaonkar -- Data collection, Analysis, Writing

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