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Isolation, Screening, Identification, and Assessment of Laccase-Producing Fungi Isolated From Different Environmental Samples

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One of the extracellular enzymes involved in ligninolytic is lacasse, secreted by soft and brown rot fungus. The class of blue copper proteins that includes laccases consists of N-glycosylated multicopper oxidases. Ascomycetes, Deuteromycetes, and Basidiomycetes are all fungi that contain laccase; many of these are soft-rot fungi that break down lignin. Due to their wide range of substrate specificity, laccases have been the focus of extensive research over the past few decades. Their latest applications include anything from the textile pulp and paper industries to culinary applications and bioremediation techniques. Laccases are also used in organic synthesis, where phenols and amines are common substrates. Dimers and oligomers are produced due to the coupling of reactive radical intermediates in these reactions. The current investigation gathered 50 soft rot fungi, and the most incredible laccase-producing organisms in submerged fermentation were looked into. Ten of the 21 cultures displayed a reddish-brown color zone. Of these ten isolates (PTD 19, PTD 4, PP2J15, LKT 34, ITC 1, NRL 7, GOJ 7, PTD2, PP2J, and PKT12), only PP2J15 and GOJ 7 displayed the most reddish-brown color zone. The isolation of soft rot fungus, their molecular characterization, and testing for laccase production are all covered in this Paper. Talaromyces verruculosus and Cladosporium cladosporioides were identified as the PP2J15 and GOJ 7 strains based on sequence comparison and phylogenetic analysis with reference taxa.

Keywords: Guaiacol, Laccase; PDA; Telangana; Wood rot fungi.

A class of fungi known as the Basidiomycetes is renowned for producing a variety of extracellular ligninolytic enzymes. The benzenediol: oxygen oxidoreductases (EC 1.10.3.2) laccases (Lac) and different peroxidases, such as lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and versatile peroxidase (VP), are among these enzymes. While MnP is classed as a Mn (II): hydrogen peroxide oxidoreductase (EC 1.11.1.13), LiP is a diarylpropane: oxygen, hydrogen peroxide oxidoreductase (C-C-bond-cleaving) enzyme[1-3]. VP demonstrates both LiP and MnP catalytic capabilities. The enzymes under investigation are essential for the lignin breakdown in their native lignocellulosic substrates and the xenobiotic dye breakdown[4]. While some fungi that break down wood have all three types of lignin-altering enzymes, others only have one or two[5]. soft-rot fungi produce lignin-modifying enzymes as part of their secondary metabolism[6]. The enzymes discussed in this context are frequently created and secreted in response to carbon or nitrogen

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constraints[7]. High oxygen tension is often the best environment for MnP and LiP enzyme synthesis. However, agitation in liquid cultures of submerged soft-rot fungi inhibits this process. However, agitation, aromatic chemicals, or organic solvents are routinely used to increase the production of Lac[8]. In aerated cultures, without adding oxygen and subjected to shaking conditions, the enzyme VP, previously identified as a manganese-oxidizing enzyme capable of oxidizing veratryl alcohol, is produced[9]. They were investigating soft-rot fungi and their enzymes to see whether they could be used to break down various aromatic contaminants that cause environmental problems. Specifically, the pollutants examined included those found in pulp and paper mills[10], olive mill wastewater[11], polycyclic aromatic hydrocarbons[10], chlorinated phenols, polychlorinated biphenyls[12], dioxins, pesticides, explosives, and dyes[13]. It is typical for numerous isoforms of ligninolytic enzymes to be expressed in distinct taxa and culture conditions. The characteristics above are crucial for the process design and fungal treatment of effluent optimization. For various commercial applications, purified Lac, LiP, and MnP enzymes show significant potential[14]. The extraordinary capacity of fungi to synthesize different extracellular enzymes is well known. Basidiomycetes are the primary organisms that break down lignocellulose[15]. soft-rot, brown-rot, soft-rot, and litter-decomposing fungi are three frequent groups of basidiomycetous fungi that break down wood[16,17]. The only organisms that can successfully mineralize lignin are related to litter-decomposing fungi and basidiomycetous soft-rot fungi[5].

Recently, there has been a growing interest in researching the lignin-modifying enzymes in various soft-rot fungi. This interest derives from a comparative biological viewpoint and the hope of finding lignin-degrading systems that are more effective and can be applied in multiple biotechnological applications. The availability of information on the production of extracellular oxidoreductases by native fungal strains from various eco-physiological and taxonomic categories is, however, limited.

The three goals of this study were to (i) Collection of fungi from different Tunisian biotopes representing a variety of eco-physiological and taxonomic groups, (ii) To assess the ligninolytic potential of the fungal strains, (iii) To improve the cultivation conditions that favor high laccase yield from soft-rot fungi.

Table 1. Sample collection sites

Site No	Site Name	Strain Code	Source	Altitude
1	Perfect Tanners, Warangal	PTD	Leather processed effluent	17.9990° N, 79.6242° E
2	ITC, Bhadrachalam, Sarapaka	ITC	Paper and pulp-processed effluent	17.6943° N, 80.8628° E
3	Pakala Wildlife Sanctuary Forest	PKT	Leaf litter, decaying wood	17° 56' 59.99" N 79° 58' 59.99" E
4	Laknavaram Forest	LKT	Digged soil sample, decaying wood	18°3' 52.0092'' N 79°29'39.4548'' E
5	Mythri Rubber Industries, Rampoor	MRT	Excretory effluent	17.94541° N, 79.45780° E
6	Autonagar, Warangal	ATG	Dumped soil	18.00536° N, 79.59800° E
7	Nagaram Lake	NLT	Polluted water sample	18.08856° N, 79.57042° E
8	Pembathi-contaminated paddy soil,	PP2J	Contaminated soil	18.08493° N, 79.53934° E
9	Nerella forest soil	GOJ	Digged soil sample	18.15037° N, 79.57170° E

MATERIAL AND METHODS

Isolation and maintenance of Pure Culture

To isolate fungus, fifty samples of soil were dug up from each site at a depth of 5 to 10 cm, and 100 g of the sample was taken using a sterile spatula and placed in pre-sterilized zip-lock bags. In Telangana, ecological forest soil and industrial effluents were the sources of the samples (Table 1 and Fig. 1). Pure cultures of isolated fungal species were produced using a potato dextrose agar (PDA) medium. Using the dilution plate method, one gram of each soil sample was extracted and put into a test tube with 10 ml of sterile distilled water [18]. The suspension was serially diluted in sterile distilled water five times, from 10^{-1} to 10^{-5}. After vigorously mixing the soil samples, the solution was obtained. After letting the suspension stand for 20 minutes, 0.5ml of each aqueous dilution of the soil suspension was inoculated on Petri plates containing Potato dextrose agar. To prevent bacterial contamination, the PDA medium was given a trace quantity of streptomycin. Techniques like spread plate and pour plate were employed. For six days, the inoculation plates were incubated at 30 °C. To create pure cultures, several fungal colonies were sub-cultured (Table 1).

A small amount of fungus spores (or) fruit body was transferred from an active fungus culture to brand-new test tubes (screw cap or clogged with cotton or foam) or Petri dishes (covered with Parafilm to reduce drying) containing an appropriate agar medium. A culture is maintained at room temperature once it has been created. Periodically inspect cultures for contamination. Every three weeks, these are subcultured, tested for purity, and then subcultured again. Slants and plates were used to preserve pure cultures. There were 21 pure cultures recovered from the 50 samples collected throughout different regions in Telangana. The laccase enzyme was estimated using these pure cultures. The diverse collection of organisms known as soft-rot fungi can break down lignin, other types of wood, contaminated

Fig. 1. Sample collection from various Environmental sites

soil, and phenolic chemicals. The non-specific and non-stereo selective extracellular enzyme systems are responsible for the capacity to break down lignin. Laccases make for the extracellular enzyme system that breaks down lignin. Because the essential elements of the whit-rot lignin-degrading system are extracellular, the fungi can break down dangerous environmental contaminants and insoluble compounds like lignin.

RESULTS

Qualitative assay for Laccase enzyme

To choose appropriate generating strains, screening laccase enzyme-producing soft rot fungus species was crucial. Due to the many characteristics involved, screening for oxidative enzymes or mediators necessitates the examination of numerous samples. This is why the current method employs low-cost, quick, and sensitive testing techniques. The screening technique must be designed to find fungal strains and usually functioning enzymes. Using a guaiacol plate assay, the 21 pure cultures were examined for the efficiency of their laccase activity. Guaiacol (0.02%) was added to the Potato Dextrose Agar medium, which was then incubated for six days at 30°C with pure colonies. This laccase enzyme estimate is qualitative. Guaiacol contained in the media was able to be oxidized by soft rot fungus that generated the laccase enzyme, producing a reddish-brown zone (Fig. 2). Fig. 3 demonstrated that the absence of a reddish-brown color zone in the medium suggests that the laccase enzyme

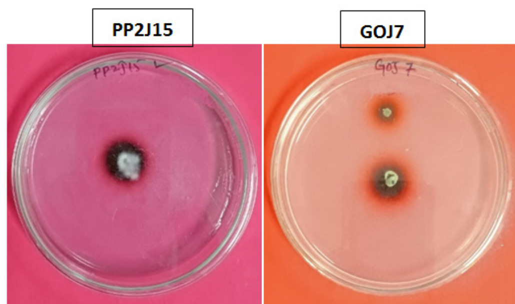

Fig. 2. Positive

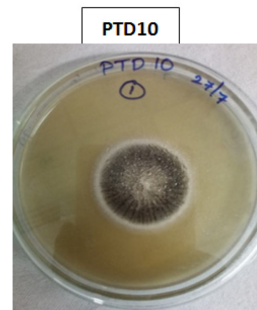

Fig. 3. Negative

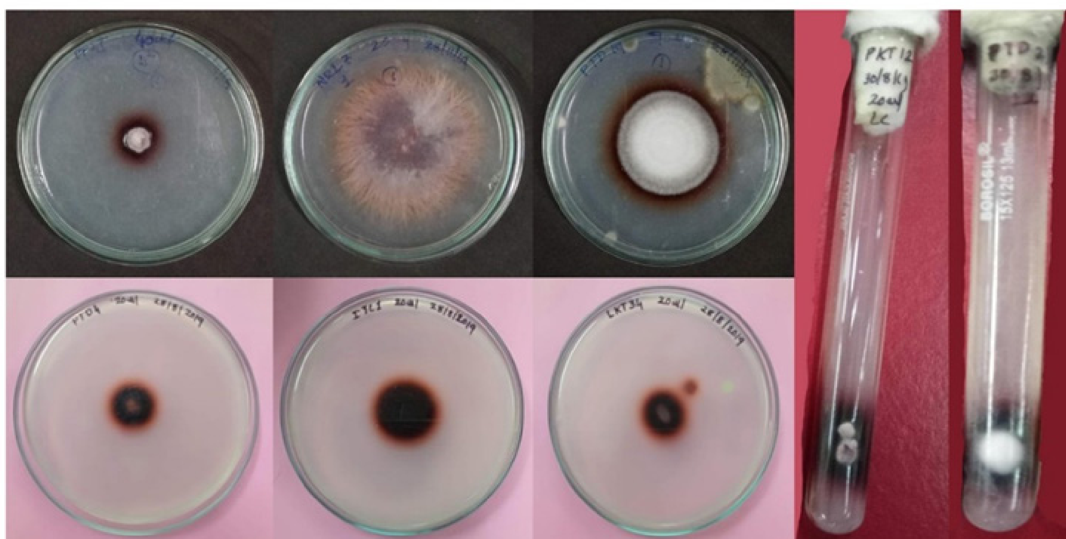

Fig. 4. Screening of Laccase-producing fungi by guaiacol plate technique

Table 2. Qualitative assay of laccase enzyme by soft rot fungi

No	Fungi	Conc. of Guaiacol (µl)	3rd DAY Diameter of reddish-brown zone (mm)	6th DAY Diameter of reddish-brown zone (mm)
1	PTD 19	20µl	3mm	5mm
2	PTD 4	20µl	4mm	6mm
3	PP2J15	20µl	7mm	10mm
4	LKT 34	20µl	3mm	5mm
5	ITC 1	20µl	5mm	6mm
6	CM10	20µl	—	—
7	NRL7	20µl	1mm	3mm
8	PM5J	20µl	2mm	4mm
9	CM1	20µl	3mm	4mm
10	PTD5	20µl	—	—
11	GOJ7	20µl	6mm	9mm
12	PTD2	20µl	2mm	3mm
13	LKT11	20µl	1mm	2mm
14	LKT10	20µl	4mm	6mm
15	PTD6	20µl	—	—
16	CM3	20µl	2mm	5mm
17	PP2J	20µl	3mm	7mm
18	MRT2	20µl	—	—
19	ITC12	20µl	2mm	3mm
20	PKT12	20µl	3mm	6mm
21	ATG5	20µl	4mm	6mm

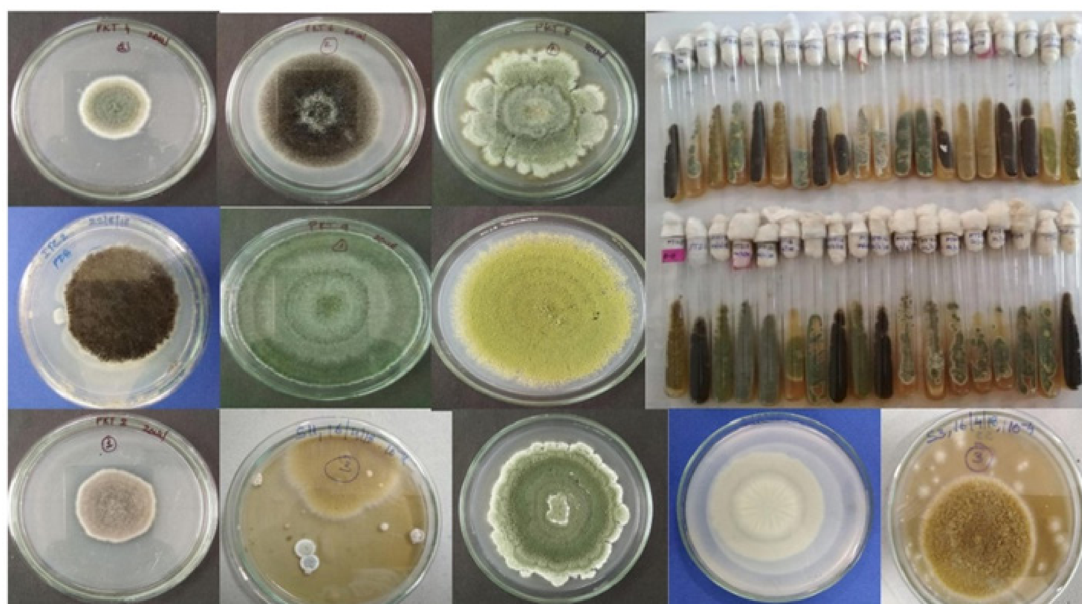

Fig. 5. In the medium, the reddish-brown color zone has not formed

synthesis is not taking place. The zone was measured and shown in Table 2 for comparison. It was clear from the table that ten fungal cultures out of the 21 pure cultures displayed a reddish-brown color zone on medium. This suggests that guaiacol is utilized as a marker for extracellular oxidative enzymes, which is supported by the current finding. Ten of them (PTD 19, PTD 4, PP2J15, LKT 34, ITC 1, NRL 7, GOJ 7, PTD2, PP2J, and PKT12) had the reddish-brown color zone from the start. The red-brown zone in the soft rot fungal cultures was highest in PP2J15 at 7 mm at three days and 10

Table 3. Quantitative assay of laccase enzyme by collected soft rot fungi

No	Fungi	pH		Laccase U/ml	
		6th	12th	6th	12th
1	PTD 19	4	4.3	211	36
2	PTD 4	5	3.6	66	121
3	PP2J15	5	5.5	625	712
4	LKT 34	4.5	4.5	76	56
5	ITC 1	5	5	152	182
6	CM10	3	4	—	—
7	NRL7	5	4.5	136	98
8	PM5J	3	5	12	2
9	CM1	5	5.8	22	46
10	PTD5	3.5	4.1	-	-
11	GOJ7	4	5.1	273	512
12	PTD2	5	4.5	62	74
13	LKT11	4	4	144	-
14	LKT10	5	5.5	-	-
15	PTD6	4	3.2	38	67
16	CM3	5	4.5	-	-
17	PP2J	3	3.5	124	4
18	MRT2	5	4	46	98
19	ITC12	5	4	36	48
20	PKT12	5	5.5	197	149
21	ATG5	5	5	-	-

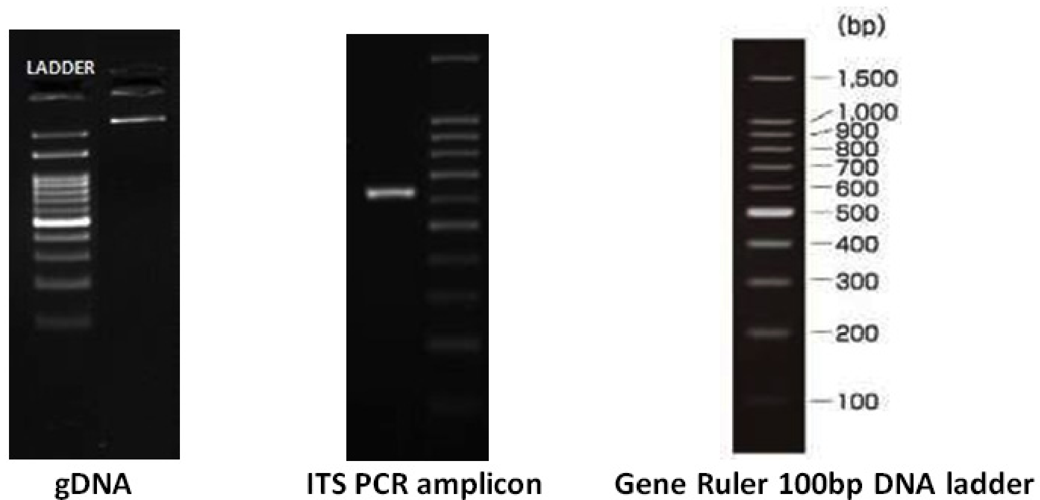

Fig. 6. gDNA and ITS Amplicon QC data (*Talaromyces verruculosus* (OR782472.1)

mm at six days, whereas GOJ7 displayed reddish-brown zone measurements of 6 mm at three days and 9 mm at six days.

Nayak et al. (2022) adopted a similar strategy and employed the guaiacol plate assay method for qualitative screening. Fungal isolates were point inoculated onto the corresponding potato dextrose agar plate that contained guaiacol (0.02%) and were then incubated at 28°C for six days. The families Chaetomiaceae, Pleosporaceae, Trichocomaceae, Nectriaceae, and Ajellomycetaeae were identified by morphological research on isolated fungi [19]. The qualitative study on Potato Dextrose Agar plates with 0.02% guaiacol was reported by Anitha et al. in 2022. The plates were incubated at 30°C for five days. After five days of incubation, the laccase-producing fungal strains displayed a reddish-brown halo around the colonies on the Guaiacol-containing plates [20]. Nayak et al. (2017) qualitatively evaluated twenty-three lignocellulolytic fungi using a guaiacol plate assay on potato dextrose agar medium. Five fungal isolates out of 23 fungal strains produced the most quality laccase[21]. A similar experiment was carried out by Gnanasalomi and Gnanadoss (2013) to identify the laccase-producing fungus. It was carried out on solid media containing colored markers such as guaiacol, 2,2 -azinobis (3-ethylbenzthiazoline-6-sulphonic acid), syringaldazine, and polymeric dyes[22]. According to Desai et al. (2011), the growth of laccase-producing fungi on potato dextrose agar (PDA) media containing tannic acid and guaiacol PDA plates were observed for growth and development of reddish-brown colored precipitate in plates and observed reddish hallow zone in guaiacol PDA plates[23].

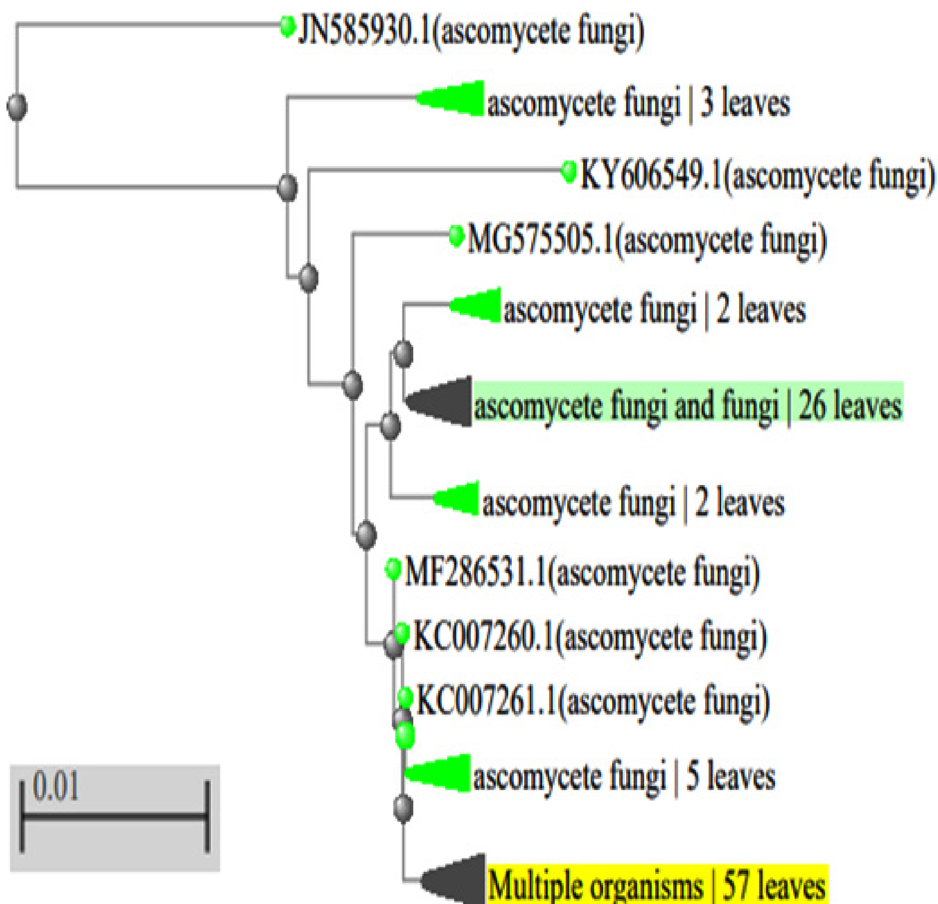

Fig. 7. Phylogenetic Tree (*Talaromyces verruculosus* (OR782472.1)

The screening is based on the indicators' color changes, which are connected to the activity of the ligninolytic enzymes. Tanwar et al. (2020) qualitative screening for visualizing corresponding hydrolysis zones of several isolates, revealing their capacity to generate diverse carbohydrates, including laccase, cellulase, and xylanase, was published in 2020. Six of the 31 isolates with cellulase activity had the largest zone size, whereas six of the 31 with xylanase activity had the largest zone size. Multiple hydrolytic enzyme activities of laccase, cellulase, and xylanase were present in ligninolytic fungal isolates, and different strains were chosen based on the zones of hydrolysis around the fungal colonies that were the highest[24].

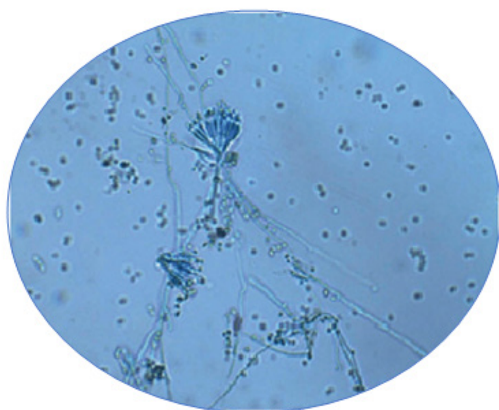

Fig. 8. Microscopic photograph *Talaromyces verruculosus* (OR782472.1)

Quantitative assay for laccase enzyme

To estimate laccase enzyme levels, the fungal isolates were cultivated in potato dextrose broth as pure cultures. For up to 12 days, pure cultures were cultured in potato dextrose broth. For the sixth and twelfth days of incubation, laccase enzyme activity was determined using a UV-visible spectrophotometer, and pH changes were noted using a pH meter. Ten of the 21 fungi examined for laccase activity in fungal cultures revealed positive results, while the other 14 were negative.

The two cultures with the highest output of laccase enzyme were PP2J15 and GOJ7 (Table 3). The laccase activity in the fungi isolates PP2J15 was notable, reaching 625 U/ml on day 6th and 712 U/ml on day 12th. GOJ7 displayed laccase activity

Table 4. Table representing the identified species with their respective accession number while sequence alignment with culture (OR782472.1)

Accession	Description
MG551571.1	Talaromyces verruculosus isolate 567
KC506174.1	Fungal sp. AM2013 strain 7
KY263515.1	Talaromyces verruculosus strain PPRI 14967
HQ657296.1	Penicillium sp. CRCF6 18S ribosomal RNA gene
KJ767054.1	Talaromyces siamensis isolate A3S2-39
MW344647.1	Talaromyces verruculosus isolate RK4Tm
OQ076506.1	Penicillium sp. isolate CF00120
KJ482652.1	Talaromyces verruculosus strain AG67
OW988114.1	Talaromyces siamensis genomic DNA sequence
OP295495.1	Talaromyces australis strain RR1331

Talaromyces verruculosus (OR782472.1) is a member of Ascomycota with the following classification.

Domain: Eukaryota
Kingdom: Fungi
Phylum: Ascomycota
Class: Eurotiomycetes
Subclass: Eurotiomycetidae
Order: Eurotiales
Family: Trichocomaceae
Genus: *Talaromyces*
Species: *T.verruculosus*

of 273 U/ml on day 6th and 512 U/ml on day 12th. For additional activities, these two cultures were utilized. On plates with guaiacol supplementation and the development of colored zones during laccase activity screening, a strong link between the two was discovered.

Pranitha et al. (2015) employed a similar strategy. Pure fungal isolates were collected and cultured in malt extract broth to determine enzyme estimates. Malt extract broth was incubated for up to 14 days after being inoculated with pure colonies. 18 of the 30 fungi tested for lignolytic

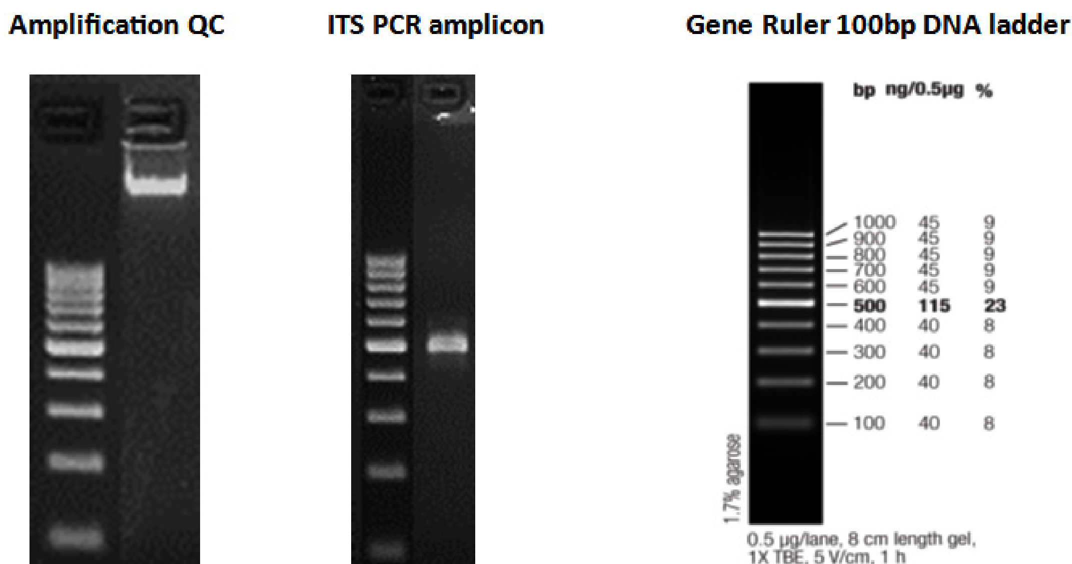

Fig. 9. gDNA and ITS Amplicon QC data *Cladosporium cladosporioides* (OR649270)

Table 5. Table representing the identified species with their respective accession number while sequence alignment with culture *(*OR649270)

Accession	Description
OR244405.1	Cladosporium cladosporioides
OR135243.1	Cladosporium cladosporioides isolate 10395-2022
OQ443091.1	Cladosporium cladosporioides isolate DHY8
MN429197.1	Cladosporium tenuissimum isolate F8144
FJ613828.1	Fungal endophyte sp. ZY-2009
MK367508.1	Cladosporium sp. isolate Z-Y-20
KU508795.1	Cladosporium cladosporioides strain CSPF3
KP689176.1	Cladosporium cladosporioides isolate FL20
ON248258.1	Cladosporium sp. isolate QTY4
KF907245.1	Dothideomycetes sp. UOM H

Cladosporium cladosporioides (OR649270) is a member of Ascomycota with the following classification.

Domain: Eukaryota
Kingdom: Fungi
Phylum: Ascomycota
Class: Dothideomycetes
Subclass: Dothideomycetidae
Order: Capnodiales
Family: Davidiellaceae
Genus*:* *Cladosporium*
Species: *C.cladosporioides*

activity out of 30 exhibited positive results, and the rest tested negative. Pv5 demonstrated, among the cultures, the most significant production of lignolytic enzymes. Pv5 showed laccase activity 350 U/ml on 7th day, 606 U/ml on 14th day, LiP activity 412 U/ml on 7th day, 544 U/ml on 14th day, MnP activity 126 U/ml on 7th day and 102 U/ml on 14th day [25]. In another study, Tapwal et al. (2014) reported four fungal isolates (ANF36, ANF212, ANF218, and ANF238) produced brown coloration around their colony and were positive for guaiacol plate assay[26]. Selvam et al. (2012) employed a novel method to confirm the lignolytic activity of the fungus. Guaiacol is one of the most often used substrates for qualitative and quantitative laccase assessment. The capacity to grow on and degrade lignin was investigated in a lignin-amended basal medium. The growth was quantified as an increase in mycelia dry weight (mg/day). The % of lignin degradation was determined to be in the range of 20.4 to 68.0, and the mycelial growth rate was in the field of 1.24 mg/day to 3.67 mg/day[27].

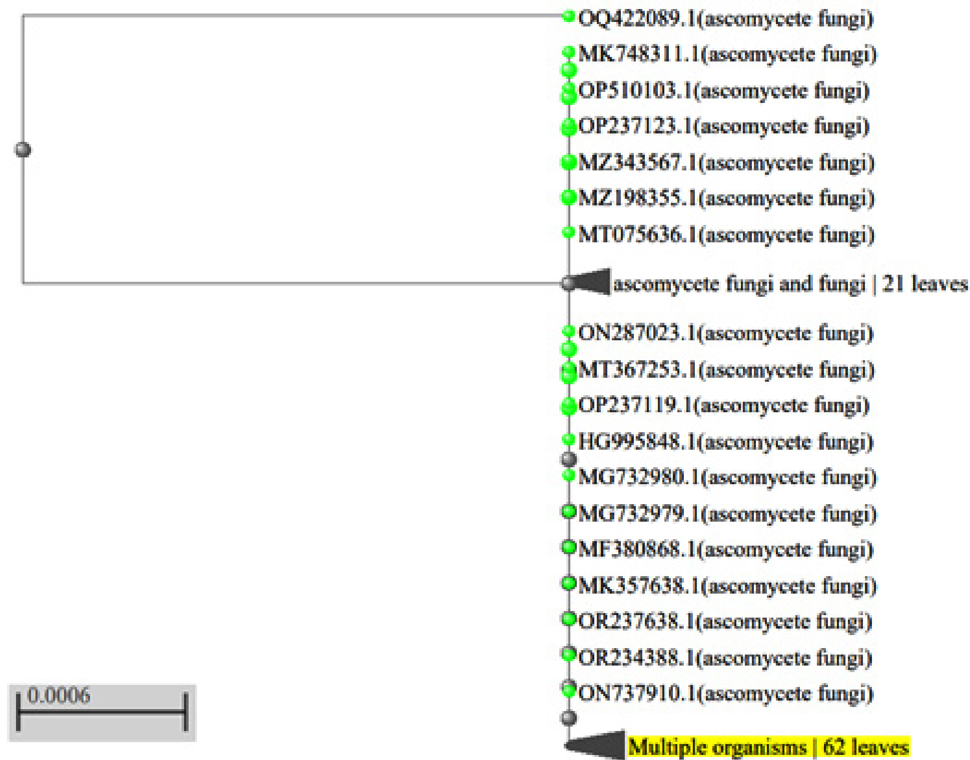

Fig. 10. Phylogenetic Tree *Cladosporium cladosporioides* (OR649270)

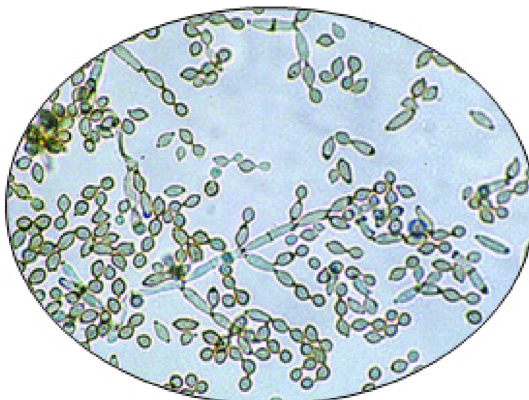

Fig. 11. Microscopic photograph *Cladosporium cladosporioides* (OR649270)

Selection of Organism

Two soft rot fungi demonstrated the highest levels of lignolytic activity according to the results of quantitative and qualitative tests, i.e., *Talaromyces verruculosus* (OR782472.1) and *Cladosporium cladosporioides* (OR649270), which were selected for further studies.

Molecular Identification

Talaromyces verruculosus (OR782472.1).

DISCUSSION

Fungi have the unique capability to efficiently break down wood components among all microbes. In the present study, various fungal strains capable of generating laccase were recovered from diverse environmental samples. The primary goals of the current study were to identify and classify laccase-producing fungi from Telangana's (Forest soil sample, industrial excretory) forest litter and industrial effluents. Twenty-one fungi were found in industrial effluents and forest trash. The preservation of microbial isolates was accomplished using Potato Dextrose Agar. To identify laccase-producing fungus derived from fungi isolated from diverse environmental samples, a straightforward screening technique was employed. This method involved the utilization of solid media containing the indicator component guaiacol. Guaiacol was used as a substrate for qualitative screening of their potential laccase manufacturing ability in the future. All of the fungal isolates were added to the potato dextrose agar plates containing 0.02% guaiacol and then cultured for five days at 30°C. The results of screening tests are displayed in Table 2. After 3 to 6 days of incubation, the fungal isolates acquired a reddish-brown halo surrounding the colonies, which indicates that laccase was produced; the absence of a reddish-brown halo indicates that laccase was not produced.

The Guaiacol plate test revealed good laccase activity in the two elite fungal isolates *Talaromyces verruculosus* (OR782472.1) (7mm on the third day, 10mm on the sixth day) and *Cladosporium cladosporioides* (OR649270) (6mm at third day, 9mm at sixth day). Incubation of the *Talaromyces verruculosus* (OR782472.1) and *Cladosporium cladosporioides* (OR649270) elite fungal isolates in Potato dextrose medium at room temperature resulted in maximal laccase production of 625 U/ml at day six, 712 U/ml at day twelve, and 273 U/ml at day six, 512 U/ml at day twelve, respectively. The molecular characterization of top fungal isolates is shown in Figures 7 and 10. Similar research was conducted by Singh et al. (2013), who also screened laccase producers like *Trichoderma harzianum* on potato dextrose agar supplemented with 0.04% guaiacol. They isolated laccase-producing fungi on PDA plates containing 0.02% of guaiacol and observed a reddish-brown oxidation zone[28]. According to Senthivelan et al. (2019), using guaiacol as a substrate, a UV-visible spectrophotometer was used to assess the laccase activity of the soft rot fungus, which was found to be 3.2 U/ml[29]. According to Monssef et al. (2016), *Trichoderma harzianum* produced laccase at a rate of 1.479 U/ml as determined by a UV-visible spectrophotometer with guaiacol as the substrate[30].

CONCLUSION

To choose strains that can manufacture laccase enzyme in the current investigation, a screening of several ambient and soil samples taken from the forest ecosystem is required. Ten isolated cultures out of the twenty-one distinct cultures were determined to be laccase-positive. In order to identify an influential laccase producer from the separate cultures, quantitative estimation was used. The isolate PP2J15 was discovered to be the most effective laccase producer. *Talaromyces verruculosus* was eventually determined to be the strain (OR782472.1) based on morphological traits and 18S rRNA gene sequencing. Maximum laccase production was recorded at days 6 and 12, respectively, and isolate (OR649270) was later determined to be *Cladosporium cladosporioides* based on morphological traits and 18S rRNA gene sequencing—maximum laccase production (273 U/ml on day 6 and 512 U/ml on day 12). A strain's potential for commercial laccase production would not solely depend on its capacity to produce the laccase enzyme. In order to increase the productivity of the target enzyme for a range of biotechnological applications, parametric optimization must be carried out to determine optimal growth and production conditions for the chosen strain. The current study also indicates the capacity of a unique ecological group of Ascomycota-phylum litter-dwelling/decomposing fungi (LDF) to produce lignin-modifying enzymes, including laccase and Mn-peroxidase. LDF was determined to be the better degrader of lignin and lignin-related chemicals despite lower titers of ligninolytic enzymes. LDF is a possible substitute for traditional soft rot fungi because they do not produce enough ligninolytic enzymes or show sufficient growth in a competitive setting like soil litter.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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