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Original Research

Isolation, Screening and Identification of Laccase Producing Fungi from Eturnagaram Forest, Warangal District, Telangana, India

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Abstract

Lacasse is one of the extracellular enzymes excreted from white and brown rot fungi, which is involved in ligninolysis. Laccases are N-glycosilated multi copper oxidases belonging to the group of the blue copper proteins. In fungi, laccase is present in Ascomycetes, Deuteromycetes, Basidiomycetes and is particularly abundant in many white-rot fungi that degrade lignin. Laccases have been subject of intensive research in the last decades due to their broad substrate specificity. In the recent years, their uses span from the textile to the pulp and paper industries, and food applications to bioremediation processes. Laccases also have uses in organic synthesis, where typical substrates are phenols and amines, and the reaction products are dimers and oligomers derived from the coupling of reactive radical intermediates. In this present study thirty white rot fungi were collected and investigated for highest laccase producing organisms in submerged fermentation. Among 30 cultures eighteen showed brown colour zone. Out of these, five isolates (Pv5, Pv3, Pv8, Pv11, and Pv12) had shown the brown colour zone from day one and Pv5 showed highest brown zone. This study describes the isolation of white rot fungi, their molecular identification and screening for their ability to produce laccase.. Based on sequence comparison and phylogenetic analysis with reference taxa the strain Pv5 was identified as *Trametes sp.*

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INTRODUCTION

The popular lignin degrading enzymes are produced mainly by white rot basidiomycetes that have the capability to degrade lignin completely to carbon dioxide and water (Reven *et al*., 2006). The lignin – degarading enzymes include lignin peroxidase (Lip), managanese peroxidase (MnP), and laccase. Among these, laccase are the most commonly produced and abundantly secreted enzymes. Laccase is a family of multicopper oxidases widely distributed among higher plants and bacteria and is found very commonly in white rot fungus. Laccase enzyme (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) is a fascinating and active member of these systems and are multi-copper enzymes belong to the blue oxidases group. They are known as oxidoreductases, which oxidizes diphenol and allied substances (Desa *et al*., 2011; Heinz *et al*., 1988; Garzil *et al*., 1998). Mostly Laccases are extracellular glyco-proteins and are multinuclear enzymes (with molecular weights between 60 and 80 kDa (Heinzkill *et al.,* 1998; Leontievsky *et al.,* 1997; Thurston, 1994). Laccase is a polyphenol oxidase which has a broad substrate specificity and efficent to degrade various phenolic compounds. For over last two decades, the applications of laccase have received huge attention from industrial and biotechnological areas. Laccase deals with wide applications of food industry, pulp and paper industry, textile industry and nanobiotechnology. White rot fungi empower laccase to degrade a wide range of pollutants which includes polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), pesticides, explosives, synthetic polymers and synthetic dyes (Pointing *et al*., 2001). Among the fungi Deutromycetes, Ascomycetes and a wide range of Basidiomycetes are known producers of laccases, which are exceptionally abundant in many lignin-degrading white-rot fungi (Bourbonnais *et al.,* 1995; Leontievsky *et al.,* 1997; Thurston, 1994). Laccases not only have the potentiality for biological delignification but it also can be applied for the treatment and detoxification of soils containing phenolic pollutants due to the broad substrate range of the enzyme (Filazzola *et al.,* 1999; Jarosz-Wilkolazka *et al.,* 2001). The main objective of the present work was to isolate potential laccase producing white rot fungi from natural habitats.

MATERIAL AND METHODS

Collection and Isolation of Fungi

Thirty different fungal fruiting bodies were collected from the tree trunks found in various parts of Eturnagaram forest, Warangal. The fruiting bodies were isolated from tree trunks and decayed wood samples. Tissue culture technique was employed for the isolation of fungi. Initially, fresh fruiting bodies were thoroughly washed under running tap water. They were then subjected to surface

sterilization with 95% ethanol for a few seconds under aseptic conditions and thoroughly washed with sterilized water. Fragments of basidiocarp were then inoculated on Malt extract agar plate and then incubated at room temperature. The cultures were purified by repeated transfer to fresh MEA (malt extract agar) plate and the pure cultures of the fungi were preserved on MEA slant culture at 4 °C.

Selection of Potent Strain

All the isolated organisms were inoculated on petri plates containing MEA medium with guaiacol and observed for development of colored zone and was assessed on daily basis. The organism showing faster growth was selected as potent strain.

Figure 1: Fruit body of *Trametes sp*

Screening of Laccase by Qualitative Assay

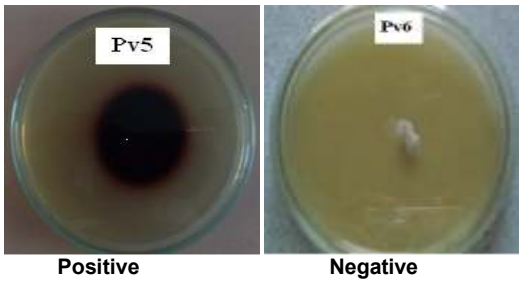
The screening is based on the colour changes of the indicators used that are associated with ligninolytic enzyme activities. Cultured organisms were grown on MEA plates containing 0.02% guaiacol and the inoculated plates were incubated at 30°C for 7 days. Out of 30 cultures tested, one culture was very effective and laccase-positive by forming reddish brown zones in the medium.

Screening of Laccase by Quantitative Assay

The pure fungal isolates were obtained and grown in malt extract broth for quantification of enzyme estimations. Malt extract broth inoculated with pure cultures was incubated up to 14 days. Out of 30 fungi tested for lignolytic activity eighteen showed positive and remaining are negative for lignolytic activity. Among the cultures Pv5 showed (Table 1) highest lignolytic enzyme production. Pv5 showed laccase activity (Table 2) 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. A good correlation was found between the formation of coloured zones screening for lignolytic activity on guaiacol supplemented plates and ligninolytic activity in quantitative assay.

Selection of Organisms

Based on the results of quantitative and qualitative tests highest laccase activity was showed by white rot fungi Pv5 was selected for further studies (Figure 2).



Positive **Negative**

Figure 2: Plates showing the positive and negative controls for Lacasse

Table 1: Qualitative assay of Laccase by white rot fungi

Fungi	Diameter of brown zone (mm)
Pv1	11
Pv2	15
Pv3	20
Pv4	6
Pv5	**32**
Pv6	--
Pv7	6
Pv8	21
Pv9	6
Pv10	--
Pv11	20
Pv12	20
Pv13	--
Pv14	--
Pv15	15
Pv16	--
Pv17	8
Pv18	--
Pv19	--
Pv20	--
Pv21	8
Pv22	6
Pv23	12
Pv24	--
Pv25	7
Pv26	--
Pv27	13
Pv28	--
Pv29	8
Pv30	--

-- Absence of the activity

Table 2: Quantitative assay of Laccase by white rot fungi

Fungi	pH		Laccase U/ml	
	7	14	7	14
Pv1	3	3.5	120	4
Pv2	4	4.5	244	36
Pv3	3.5	4.5	-	-
Pv4	5	5	228	64
Pv5	**4**	**5**	**350**	**616**
Pv6	5	5.5	-	-
Pv7	5	5.5	254	172
Pv8	4.5	4.5	76	56
Pv9	5	5.5	22	46
Pv10	5	4.5	-	-
Pv11	3	5	12	2
Pv12	3	3.5	120	4
Pv13	5	5.5	-	2
Pv14	5	5	-	-
Pv15	5	5	-	-
Pv16	3	3.5	124	4
Pv17	5	5	-	-
Pv18	5	5	-	-
Pv19	5	5	-	-
Pv20	5	5	-	-
Pv21	5	4	46	98
Pv22	5	4	36	48
Pv23	5	4.5	62	74
Pv24	5	5	-	-
Pv25	5	4.5	136	98
Pv26	5	5.5	-	-
Pv27	5	5	152	182
Pv28	5	4	-	-
Pv29	5	3.5	66	124
Pv30	5	4.5	-	-

Molecular Identification

The strain which was showing more guaiacol activity was choosen for the molecular identification and the genomic DNA of this strain was extracted using modified CTAB method. Mycelia of the species was grown on MEA culture plates were scrapped off and placed in 1.5 ml tube containing 600 µl CTAB buffer. With a sterilized pestle, the mycelia were ground to release the DNA, and then, incubated at 65^{o}C for 15 minutes in a heat block machine. Afterwards, 600 µl chloroform: isoamyl alcohol (C:IAA 24:1) was added, mixed by inverting the tube slowly for more than 50 times, and then, centrifuged at 13,000 rpm for 15 minutes at 25 ^{o}C. The upper aqueous phase was pipetted to a new tube and to this, 300 µl cold isopropanol was added and later kept in the freezer at (-20^{o}C) for 20 minutes. The tubes were again centrifuged at 13,000 rpm for 5 minutes at 4^{o}C. The supernatant was discarded and the DNA pellets was washed with 70% EtOH, and then, air dried until EtOH has totally evaporated. Finally, the DNA was diluted in 50 µl TE buffer. The genomic DNA of the macro fungi were then subjected to PCR to amplify the ITS regions of the nuclear ribosomal DNA using two primers: ITS 1 (5′TCCGTAGGTGAA CCTTGCGG 3′) and ITS 4 (5′TCCTCCGCTTATTGATATGC3′) (White *et al.,* 1990). The PCR reaction included 1 x PCR buffer, 2.5 µM MgCl2, 200 µMdNTP, 0.5 µM of each primer, 1 U Taq DNA polymerase, and 50-100 ng extracted genomic DNA and nanopure water to make a volume of 50 µl. The PCR conditions are as follows: 94 ^{o}C for 3 min, followed by 30 cycles at 94 ^{o}C for 1 min, 52 oC for 50 sec and 72 ^{o}C for 1 min, with a final extension step of 72 ^{o}C for 10 min 481 (Karunarathna *et al*., 2011). The PCR products were then purified using QIAGEN purification kit following the manufacturer's instructions and the purified PCR products were sent to Scigenome Cohin, Kerala, India for outdoor DNA sequencing. The sequences of fungus were compared using the GenBank database and basic local alignment search tools (Blast) for nucleotide analysis. The identification was confirmed with >98% similar to the fungal species deposited in GenBank. The sequence of the organisms is identified as *Trametes* sp.

RESULTS AND DISCUSSION

Thirty basidiomycetous fungi were screened for laccase activity using the guaiacol plate screening method on MEA medium. Among 30 cultures eighteen showed brown colour zone. Out of these, five isolates (Pv5, Pv3, Pv8, Pv11, and Pv12) had shown the brown colour zone from day one and Pv5 showed highest brown zone (32 mm). Metuku *et al*., (2011) screened the white rot fungi based on the polymerization of guaiacol in wood powder agar plates caused by extracellular phenoloxidases and or peroxidases excreted by the fungi. Savitha *et al.* (2011) screened laccase producing white rots on potato dextrose agar (PDA) media containing tannic acid and observed for development of brown colored precipitate in plates and observed reddish hallow zone in guaiacol containing plates. DNA was isolated from this culture and its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed.Fragment of D2 region of LSU (Large subunit 28S rDNA) gene was amplified by PCR from the above isolated plasmid DNA. A single discrete PCR amplicon band of 835 bp was observed when resolved on Agarose Gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with DF and DR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 724 bp of D2 region of 28S rDNA gene was generated from forward and reverse sequence data using aligner software. The D2 region of LSU (Large subunit 28S rDNA) gene sequence was used to carry out BLAST with the nucleotide database of NCBI genbank database. Based on maximum identity score first ten sequences in Sequence Producing (Figure 3) Significant Alignments were selected and the phylogenetic tree was constructed using MEGA 5. The evolutionary history was inferred using the Neighbor-Joining method (Saitou *et al.,* 1987).

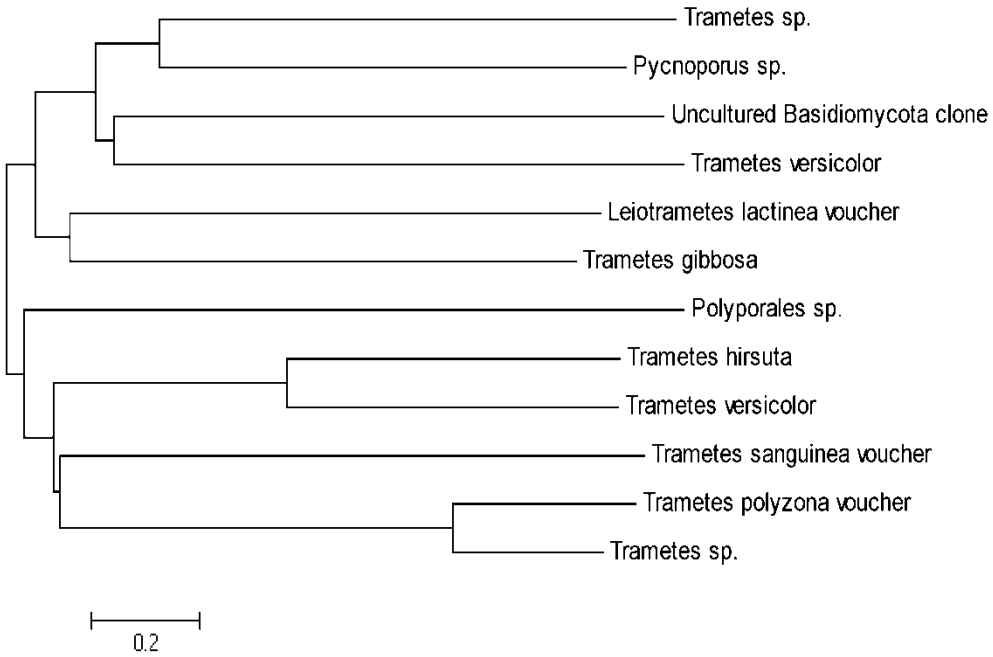


Figure 3: Phylogenetic tree was constructed using MEGA-5 (Molecular Evolutionary Genetics Analysis) software by the neighbor joining method

The optimal tree with the sum of branch length = 11.54906060 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 839 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.,* 2011). The culture, which was labeled as Pv5 was found to be *Trametes sp* based on nucleotide homology and phylogenetic analysis.

CONCLUSIONS

The present research work aimed at isolation of laccase producing fungi their molecular identification and screening for their ability to produce laccase. In conclusion, the white rot fungus *Trametes sp.* was very successful in production of extracellular lignolytic enzymes. Laccase producing fungi have many biotechnological applications and the isolated fungi might be used for further research projects and appeared to be a good source for industrial application in biobleaching.

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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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