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MOLECULAR IDENTIFICATION OF *PLEUROTUS* SPP ISOLATED FROM YELAGIRI NICHE AND ITS LACCASE PRODUCTION

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ABSTRACT: Laccase (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductase) are a group of multi-copper containing enzymes mostly produced by group of white rot fungi. In the present study we have isolated and identified as potential laccase producing *Pleurotus ostreatus* and *Pleurotus cystidiosus* from Yelagiri Hills and the gene bank accession number were MH591762 and MH591763. The laccase production was screened using plate assay method and *P.cystidiosus* was found to be the maximum producer, it's a first report for the production of laccase.

KEYWORDS: Polymerase Chain Reaction, Phylogenetic Analysis, *Pleurotus ostreatus, Pleurotus cystidiosus.*

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1. INTRODUCTION

Laccases are belonging to the group of multi-copper proteins that oxidize various aromatic and non-aromatic compounds by molecular oxygen act as terminal electron acceptor [1]. They have been widely distributed in nature and are found in plants, insects, bacteria [2], fungi and actinomycetes [3]. The fungal laccase are prominent in white rot basidiomycetes which are involved in lignin degradation. Some of the most important fungi are *Pleurotus ostreatus* [4]. Discovery of novel laccase is important in enzyme technology for biotechnological applications [5]. It covers the production of wide range of our desired products used in industries like food, animal feed, pharmaceuticals, fine and bulk chemicals, fibers, hygiene, and environmental technology, and

analytical purposes [6]. During the past 50 years this was using as biocatalysts in many process, biosynthesis, molecular biology, for the modification and optimization for new synthetic schemes and the solution of analytical problems. Laccase have the ability to oxidize both phenolic and non-phenolic compounds as well as highly recalcitrant environmental pollutants, which makes them very useful for their application to various biotechnological processes such as textile dye bleaching [7], pulp bleaching [8], effluent detoxification [9], anti-cancer drugs, cosmetics [10] and bioremediation of contaminating environmental pollutants, food industry, Enzymatic conversion of chemical intermediates and organic synthesis [11]. The objective of the present study was to isolate, identify, and detect the laccase enzymes secreted by these ligninolytic fungi collected from the Yelagiri Hills, in Vellore, Tamil Nadu, India.

2. MATERIALS AND METHODS

2.1. Collection of Samples

The fruit bodies were collected from the tree barks in Yelagiri Hills. The geographical location of the study was carried out in Latitude of 12.606376^0N and Longitude of 78.626890^0E of India. The tree bark sample was collected by using a sterile container and brought to the laboratory and sub cultured on Potato Dextrose Agar (PDA). The mycelia were washed thoroughly with sterile water and small fragments of basidiocarp was then inoculated on PDA plate and incubated at 30^0C for 5 to 10 days. The purified cultures were repeatedly transferred to freshly prepared PDA slant and preserved at 4°C for further studies.

2.2. Screening for Laccase Production by Plate Assay

The laccase screening method was adopted from the protocol described by [12], with minor modifications. The isolated fungal culture was screened for laccase production on PDA media incorporated with tannic acid and guaiacol. The fungal mycelia (2mm) disk were inoculated and incubated at 27ºC for five days.

2.3. Molecular Identification of Organisms

2.3.1. Isolation of DNA from *Pleurotus* spp

DNA was isolated using [13] method. Around 500mg of mycelium of two fungi were collected using sterile scalpel from the medium and put into 2.0 ml fresh tubes and then ground into fine powder for 5 min. The crushed mycelia were resuspended in 600ul ml extraction buffer (100mM Tris-HCl (pH 8), 2% (W/V) CTAB, 50 mM EDTA, 0.7 M Nacl, 1% (V/V) β mercapto ethanol and 1% (W/V) PVP) and incubated for 1 hr at 65°C. Solution (0.5ml) of chloroform iso amyl alcohol (24:1 V/V) was added into the mixture of extraction buffer and the two phases were mixed several times by inverting tubes gently. The resulting emulsion was centrifuged at 12,000rpm, 4°C, for 5 min using REMI Cooling centrifuge (RM24). The upper aqueous phase was mixed with 50µL of NaCl and 400µL of isopropanol in 2.0ul tubes. Samples of DNA were left to precipitate for 1h at 4ºC and centrifuged at 10,000rpm, 4ºC for 5 min. The supernatant was discarded and pellets air- dried and

washed two times with an equal volume of 70% ethanol. Pellets of DNA were then resuspended 10 50ul of 1xTE buffer. Purified DNA was then stored at -20ºC. DNA was confirmed using 0.8% agarose gel electrophoresis.

2.3.2. Polymerase chain Reaction

For the ITS region amplification, the ITS1 forward (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 reverse primers (5′-TCCTCCGCTTATTGATATGC-3′) were used. The PCR reaction mixture as follows; was carried out in a 50 µL total volume reaction consisting of 25.0 µL PCR master mix (SIGMA), 2.0 µL of both primers, 5.0 µL of template DNA and 16 µL nuclease free water. The PCR program conditions were as follows: 94°C for 5 min for initial genomic DNA denaturation, 30 cycles of 94°C for 40 s, 55°C for 30 s, and 72°C for 1 min, and a final elongation step at 72°C for 5 min. [14]. The PCR products were analysed to test the integrity and concentration on 1.5% agarose gel using electrophoresis. PCR products were directly sequenced from both directions using the same primers used for the amplification. The final cleaned PCR products were stored in -4ºC for further gene sequence.

2.3.3. Phylogenetic Analysis

The PCR product were sent to Chromous Biotech (Bangalore) and were sequenced using an ABI 3730XL capillary electrophoresis sequencing station (Applied Biosystem, USA). BLAST alignment search tool of NCBI gen bank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using software MEGA 5.

3. RESULTS AND DISCUSSION

3.1. Morphology Identification of *Pleurotus* spp.

In the present study four individual laccase producing fungi belonging to basidiomycetes group were isolated from collected tree barks sample. The figure 1 shows the location of sample collection from Yelagiri Hills and isolated two potent laccase producer *Pleurotus ostreatus* and *Pleurotus cystidiosus*. The four fungal isolates are identified as individual *Pleurotus* spp based on the macroscopic and microscopic examination on the surface texture [15], pigmentation [16] and spores at the hyphal tips which were used to identify that species level using standard manual [17].

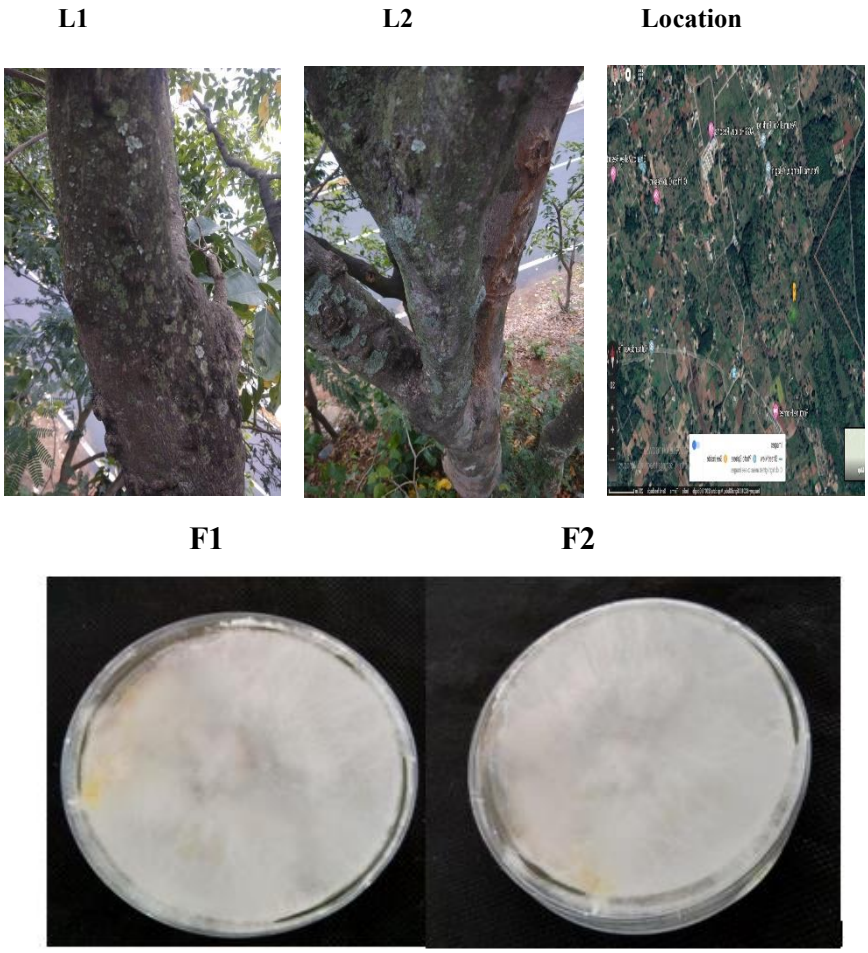

Figure 1: *Pleurotus ostreatus* and *Pleurotus cystidiosus* culture on PDA Media

3.2. Screening of Laccase Production

The incubated plates show the development of brown colored precipitate in tannic acid plates and reddish hallow zone in guaiacol plates around the fungal colonies in the PDA plate. Among the 4 individual fungal isolates *P.ostreatus* and *P.cystidiosus* are more potent laccase activity when compared with others. The *P.ostreatus* and *P.cystidiosus* were used for further studies. [18] Reported that white rot fungus *Phanerochaete chrysosporium* belonging to basidiomycetes family, having a high peroxidative activity. [19] Demonstrated to isolate and identified the lignin degrading white rot fungi from wood decay sample by using plate assay method. [20] Investigated to screen a fungi *P.chrysosporium, P.ostreatus, Auricularia auriculajudae*, and *Lentinus edodes* by using Dimethylsuccinate (DMS) medium and the laccase activity was measured.

3.3. Molecular Identification of Organisms

3.3.1. Isolation of DNA from *Pleurotus* spp

The Isolated genomic DNA of *P.ostreatus* and *P.cystidiosus* were confirmed by 1% agarose gel electrophoresis was shown in Figure 2. [21] Reported the *Marasmius quercophilus* genomic DNA was prepared and cloned the structural gene lac1 in *Escherichia coli*. [22] isolated a genomic DNA

of *Trametes villosa,* it was identified as Icc1 and Icc2 laccase genes, cloned and sequenced. [23] Studied Icc3 Icc4 and Icc5 laccase gene family was isolated from *T.villosa* from genomic DNA.

Figure 2: Genomic DNA from samples loaded in 1% agarose gel F1- *P.ostreatus* and F2 – *P.cystidiosus*

3.3.2. Polymerase Chain Reaction (PCR)

The isolated genomic DNA of fungal species were confirmed by gel electrophoresis with standard molecular markers was shown in figure 3. [24] Reported that, five newly identified laccase gene coding sequences with 95-99% similarity in nucleotide and amino acid sequence in the genome of *Trametes versicolor.* Several laccase genes was isolated and characterized recently, and in some cases, these sequences are multigene families, coding for more than one non-allelic variant [25]. The different white rot fungal laccase gene was studied such as Lcc1 gene from *Trametes trogii* [26], *Lentinus triginus* [27], *Pycnoporus cinnabrinus* [28] and *T.versicolor* [29]. Lac2 gene from *Coriolopsis caperata* [30], *Coriolopsis gallica* [31], Lac gene from *Trametes hirsute* [32].

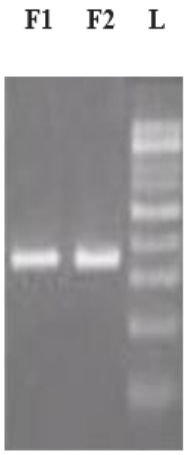

Figure 3: PCR amplification of 18s rRNA fragment from fungal sample. The size of PCR amplified product is ~1.5-1.8kb respectively. Lane Description F1- *P.ostreatus* and F2 – *P.cystidiosus* and L-500bp DNA Ladder

3.3.3. Phylogenetic analysis

The PCR amplicons sequences were found to be 97 – 100% similar sequences of 18s rRNA regions of the respective genera and species of closely related fungi documented in the GenBank accession number MH591762 and MH591763. These findings allow us to infer homology from the degree of similarity found between our isolated and those within the GenBank database. The 18s rRNA sequences results were used to constructed phylogenetic tree on Mega 5.0 software (Figure 4).

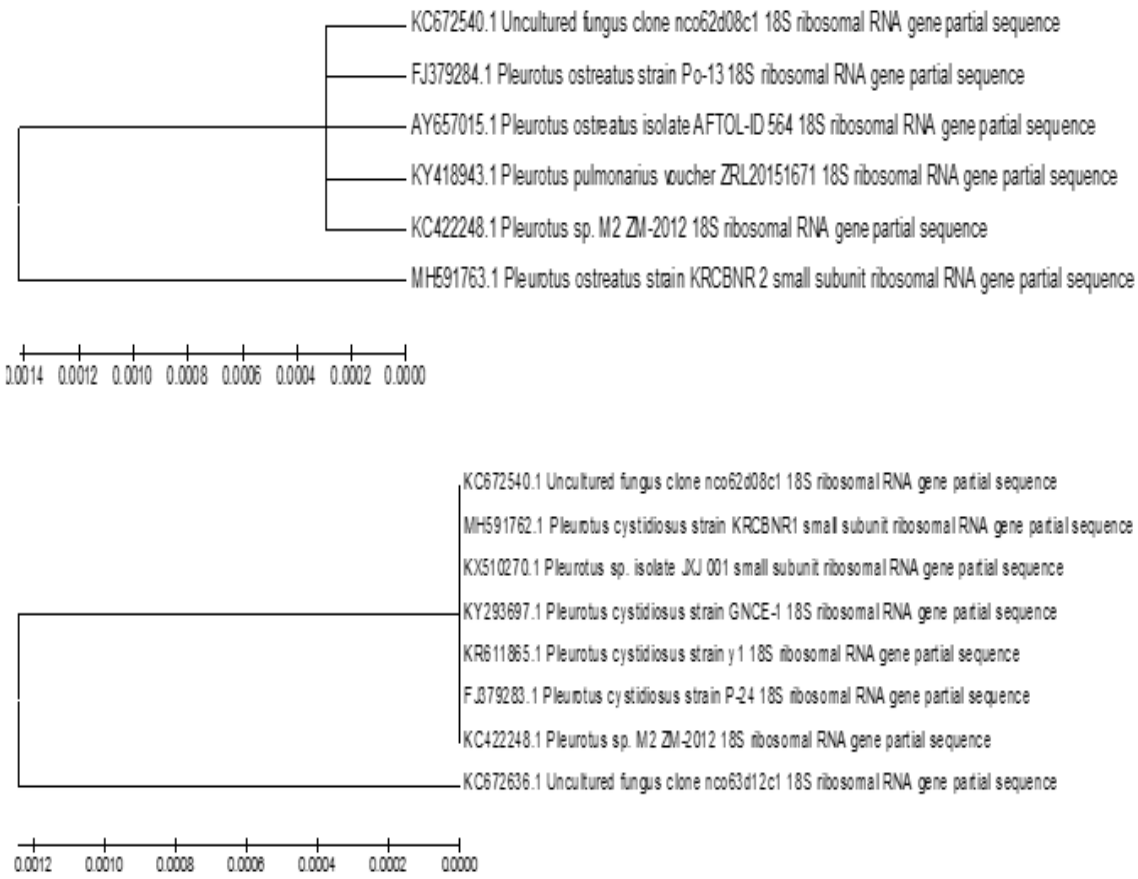

Figure 4: The phylogenetic tree of the *P.ostreatus* and *P.cystidiosus* that were isolated from tree bark Yelagiri Hills, in Vellore Tamil Nadu, India

The gene sequence was successfully used for phylogenetic analysis at higher taxonomic levels within the Basidiomycota in previous studies [33]. Studies on phylogenetic reconstructions indicate that sequence diversity between fungal laccases is moderate and that the isoforms described to date originates from the same common ancestor [34].

4. CONCLUSION

The present investigation of four different *Pleurotus* spp strains was isolated from Yellagri Hills from tree bark, the *P.ostreatus* and *P.cystidiosus* was chosen because it exhibited a fast and large oxidation of guaiacol on agar plate. The Genomic DNA of both fungal species was isolated and confirmed by gel electrophoresis. In PCR forward and reverse primers was used to sequence the

specified gene and submitted to NCBI. The sequenced gene was identified and GenBank accession number for *P.cystidiosus* MH591762, and *P.ostreatus* MH591763. It has 97% similarity of 18s rRNA sequence

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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