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Molecular characterization and phylogenetic analysis of laccase producing fungal isolates with dye decolourizing potential

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Twenty six fungi were isolated and screened for their ability to produce laccase on solid medium containing guaiacol and ABTS. Among these, six isolates showed positive laccase activity. They were quantitatively screened and the best two cultures were selected for further investigation. The two cultures also exhibited good dye decolourization ability. Molecular characterization based on the ITS rDNA region were carried out for the two cultures. The strains were identified as *Psathyrella candolleana* LCJ 178 and *Myrothecium gramineum* LCJ 177 (GenBank accession numbers KF414680 and KF414681) respectively. These cultures showed the highest laccase production under submerged fermentation. This paper for the first time reports the production of laccase using *Myrothecium gramineum* and its ability to degrade dyes.

Keywords: laccase, molecular identification, dye decolourization, ITS region

Laccases (benzenediol: oxygen oxidoreductase EC 1.10.3.2) are blue copper-containing enzymes that catalyze the oxidation of various substrates and use molecular oxygen as an electron acceptor (Revanker and Lele, 2006). These proteins are characterized by the presence of four catalytic copper atoms. Laccases are distributed widely in a wide range of living organisms like higher plants and, insects (Hattori *et al.,* 2005) and bacteria (Claus, 2004). Laccases have been reported in fungi belonging to Ascomycetes, Deuteromycetes and Basidiomycetes. Fungal laccases have higher redox potential and are involved in the degradation of lignin and toxic phenols. Fungal laccases therefore have great potential in many industrial applications like paper, textile, food and pharmaceutical sectors and in the degradation of aromatic pollutants causing environmental problems (Sette *et al.,* 2008). Fungi that possess ligninolytic ability are capable of degrading screening reagents with a structure similar to lignin. Screening of laccase producing fungi is done on solid media containing coloured indicators like guaiacol, 2,2 -azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), syringaldazine and polymeric dyes (Tapia-Tussell *et al.,* 2008). The screening is based on the colour changes of the indicators used that are associated with ligninolytic enzyme activities. Identification and characterization of strains is based on morphological and physiological methods which are time-consuming (Fleet, 1992). This method of identification is non reliable since the morphological characteristics varies with different media used (Brandt *et al.,* 1998). Morphological and biochemical characteristics of fungi are used for identification universally, but distinguishing between closely related

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fungi requires molecular techniques (Shahriarnour *et al.,* 2011). DNA-based identification methods are reliable and faster than phenotypic characterization (Kurtzman *et al.,* 2003). PCR-based methods are used for identification at the genus and species level (Chen *et al.,* 2001).

Decolourization of textile dyes is a serious problem and treatments using biological methods are cost effective and easy to use. Dye decolourization using enzymes like laccases has been suggested as an eco-friendly alternative for treatment of dyes (Robinson *et al.,* 2001). However, yield of fungal laccases produced for industrial and commercial purposes are limited (Hong *et al.,* 2006). Screening of new laccase producing fungi that are efficient and secrete high yields of laccase are necessary. In the present study, the isolation and screening of fungi for laccase production has been carried out. The ability of selected fungi on the decolourization of various textile dyes was also investigated.

Materials and methods

Fungal collection

Fruiting bodies of mushroom used for the isolation were collected from Loyola College Campus, Chennai, India. Mycelium was isolated by aseptically transferring the upper unexposed part of the basidiocarp on Potato Dextrose Agar (PDA). The plates were incubated at 28 - 30 °C for 7-10 days under dark conditions. Distinct fungal colonies were isolated and repeatedly sub-cultured until pure cultures were obtained. The cultures were maintained on PDA slants at 5 °C.

Primary screening (Qualitative) for laccase activity

Qualitative screening method for laccase production were carried out by inoculation of 1 cm diameter of mycelium from each strain onto PDA plates containing 4 mM Guaiacol and 2mM ABTS and then incubated at 30 °C. The formation of reddish brown halo in guaiacol supplemented plates and dark-green halo in the ABTS supplemented plates indicated a positive laccase secretion. The diameter of the halo indicated the level of laccase produced.

Secondary screening (Quantitative) for laccase production

Secondary screening of laccase positive cultures was carried out in 250 mL Erlenmeyer flasks containing 100 mL of Potato dextrose broth. Two mycelial agar plugs (4 mm) from PDA plates were used to inoculate the production. Flasks were sterilized at 121 ^{o}C for 15 min and incubated with shaking at 120 rpm at room temperature. Sampling was done at regular intervals for laccase activity and all the experiment was carried out in duplicates.

Enzyme assay

Laccase activity was measured spectrophotometrically using guaiacol as a substrate with an absorbance coefficient value of 6800 $M^{-1}cm^{-1}$ at 470 nm (Collins and Dobson, 1997). The reaction mixture consisted of 3 mL of 100 mM of guaiacol dissolved in 10% acetone (v/v) in sodium acetate buffer (100 mM, pH 5.0), and 1 mL culture filtrate. The mixture was incubated for 15 min and the absorbance was read at 470 nm. One unit (U) of laccase activity was defined as the amount of enzyme catalyzing the production of one micromole of coloured product per min per mL.

Calculation

Laccase activity (U/mL) =

$$\frac{\Delta A470/min \times 4 \times Vt \times dilution\ factor}{€ \times Vs}$$

Where

Vt = final volume of reaction mixture

Vs = sample volume

€ = extinction coefficient of guaiacol = 6740 $M^{-1}cm^{-1}$

4 = derived from unit definition and principle

Qualitative analysis for dye decolourizing ability

The decolourizing potential of the isolated fungi was evaluated by inoculating the isolated fungi on PDA plates containing

0.02% brilliant green, methyl red, bromophenol blue, crystal violet, erichrome black and phenol red. Fungal cultures about 1 cm agar disc was inoculated on the plates and incubated for 10 days. Formation of zone of clearance under and around the fungal colony indicated the decolourization potential of the isolated cultures (Dhouib *et al.,* 2005). Dye plates that were not inoculated served as the control.

Molecular identification

The cultures showing high laccase activity were genotypically identified. Isolation of genomic DNA was carried out by Genei genomic DNA kit. The purity of the DNA was calculated at 260/280 nm and this was further used for Polymerase chain reaction (PCR). Sequencing was carried out at Solgent, Korea. The PCR amplification of 18S rDNA was carried out in the ITS1-5.8S-ITS2 region of the rDNA of the selected fungi was amplified using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) primers (Bakri *et al.,* 2010). The nearest fungus with similar sequence was evaluated with GenBank database using BLAST with our sequence as the query sequence (Altschul *et al.,* 1997). Alignment with the similar sequences was carried out using ClustalW (Thompson *et al.,* 1994). Phylogenetic tree was structured by neighbour tree joining method with MEGA 5 software (Tamura *et al.,* 2007).

Results and discussion

Isolation and primary screening of laccase producing fungi

Around 26 different fungi were isolated by the following standard protocol. The twenty six isolated cultures were screened for laccase production by inoculation in PDA plates containing 4 mM guaiacol and 2 mM ABTS. Among the 26 isolates 6 were found to be laccase positive by the formation of reddish brown halo under and around the colony in Guaiacol supplemented agar and dark-green halo in ABTS supplemented plates was considered as a positive reaction for laccase activity (Table 1). Guaiacol and ABTS were considered as the best substrates for laccase activity (Thurston, 1994). Reddish brown colour and green colour around the colonies were due to the oxidative polymerization of guaiacol and ABTS in the presence of extracellular fungal laccase. The intensity and diameter of the halo indicates the level of laccase enzyme secreted (Table 2). Coloured indicators are used for the visual recognition of laccase enzyme which is a direct method of enzymes screening as measurement is not necessary (Machado *et al.,* 2005). The isolates which did not show any colour change lack laccase activity and were not considered for further work.

Table 1: Primary screening for laccase activity in the fungal isolates

Isolate No	Laccase activity
LCJ 177	**+**
LCJ 178	**+**
LCJ 179	-
LCJ 180	-
LCJ 181	-
LCJ 182	-
LCJ 183	-
LCJ 184	-
LCJ 185	-
LCJ 186	-
LCJ 187	**+**
LCJ 188	-
LCJ 189	**+**
LCJ 190	**+**
LCJ 191	**+**
LCJ 192	-
LCJ 193	-
LCJ 194	-
LCJ 195	-
LCJ 196	-
LCJ 197	-
LCJ 198	-

Table 2: Primary screening (Qualitative) for Laccase positive isolates using guaiacol and ABTS

Isolate No	Habitat	Guaiacol	ABTS
LCJ 177	Soil	++++	++++
LCJ 178	Soil	+++++	++++
LCJ 179	Soil	-	-
LCJ 180	Bark	-	-
LCJ 181	Soil	-	-
LCJ 182	Soil	-	-
LCJ 183	Bark	-	-
LCJ 184	Bark	-	-
LCJ 185	Bark	-	-
LCJ 186	Soil	-	-
LCJ 187	Bark	+++	++++
LCJ 188	Bark	-	-
LCJ 189	Bark	+++++	++++
LCJ 190	Soil	+++	++
LCJ 191	Soil	++	++++
LCJ 192	Bark	-	-
LCJ 193	Bark	-	-
LCJ 194	Bark	-	-
LCJ 195	Bark	-	-
LCJ 196	Bark	-	-
LCJ 197	Bark	-	-
LCJ 198	Soil	-	-

Key: +++++ - excellent, ++++ - very good, +++ - good, ++ - average, + - poor, - none

Similar studies were carried out by Tapia-Tussell *et al.* (2011) who isolated new fungal isolates with laccase activity and potential in dye decolourization. Poojary *et al.* (2012) also isolated novel basidiomycetes with dye degrading ability.

Secondary screening of laccase activity

The laccase production of the six laccase positive cultures is shown in Table 3. Secondary screening experiments with the selected isolates showed that the highest levels of laccase production were produced by LCJ 177 and LCJ 178, while the other cultures did not produce significant amount of extracellular laccase. The cultures that showed positive reaction on the guaiacol and ABTS plates but did not produce laccase in the liquid medium may have produced other ligninolytic enzymes (Ryu *et al.,* 2003). The enzyme activity was measured every alternative day and continued till decrease in laccase activity was observed. The laccase activity was maximum on the 6th day of incubation. Secondary screening of enzyme activity in a liquid medium is essential because extracellular enzymes like laccase are produced during the log phase of the organism. Kiiskinen *et al.* (2004) conducted a similar study with the isolated fungi and showed that laccase activity is dependent on the liquid medium. Colao *et al.* (2003) also showed that laccase production by fungi depends on the cultivation medium.

Table 3: Secondary screening (Quantitative) for Laccase positive isolates

Isolate No	Laccase activity (U/mL)
LCJ 177	**0.62**
LCJ 178	**0.92**
LCJ 187	0.25
LCJ 189	0.07
LCJ 190	0.38
LCJ 191	0.06

Table 4: Qualitative assay for dye decolourization ability

Isolates	Brilliant green	Methyl red	Bromophenol blue	Erichrome Black	Crystal violet	Phenol red
LCJ 177	++++	+	++	++	++++	-
LCJ 178	+++	+	++++	++	+	-
LCJ 187	+++++	+++++	+	-	-	-
LCJ 189	-	-	-	-	-	-
LCJ 190	+	+++	++	-	-	-
LCJ 191	-	-	-	-	-	-

Key: +++++ - excellent, ++++ - very good, +++ - good, ++ - average, + - poor, - none

Qualitative analysis for dye decolourizing ability

The dye decolourizing ability of the laccase producing fungi was carried out. The plates were observed for the decolourization rates after 10 days of incubation. Among the six fungal isolates tested LCJ 178 and LCJ 177 decolourized most of the dyes faster. The other cultures showed less or no ability to decolourize the dyes (Table 4). Both LCJ 177 and LCJ 178 however did not completely decolourize the dyes at the end of 10 days. Poojary *et al.* (2012) conducted a similar study and isolated two fungal strains capable of dye decolourization. Another study by Levin *et al.* (2004) also shows the dye decolourizing ability of fungi isolated in Argentina. Decolourization of textile dyes by a *Clitocybula dusenii,* white-rot fungus producing laccase was reported by Wesenberg *et al*. (2002). Liu *et al.* (2004) also reported that laccase is solely responsible for the decolourization and degradation of dyes.

Molecular identification

DNA isolation and sequencing

Among the 26 isolates, LCJ 177 and LCJ 178 exhibited high laccase acivity and dye decolourizing ability. Hence they were subjected to molecular identification. Isolation of genomic DNA for the selected fungal isolates was carried out. PCR-amplification of the genomic DNA at the ITS region of the rDNA was carried out using the universal primers (ITS1 and ITS4). A clear taxonomic resolution is achieved by ITS sequences than sequences generated from coding regions (Anderson *et al.,* 2003). In the genome of fungi the ITS region is present in a large number of copies as part of the tandemly repeated nuclear rDNA. This ITS region when coupled with PCR amplification forms a sensitive assay (Jasalvich *et al.,* 2000). The amplified products were 709 bp in length for *Psathyrella candolleana* LCJ 178 and 597 bp for *Myrothecium gramineum* LCJ 177. Similar sequences were collected using BLAST program in NCBI database and the phylogenetic tree constructed. Based on the BLAST search of the complete sequence of ITS region the two selected fungi were found to be in closest homology of 99% with *Psathyrella candolleana* (Fig. 1) and *Myrothecium gramineum* (Fig. 2). The sequences of the isolates were deposited in the GenBank database and assigned with respective accession numbers. The selected isolates were identified as *Psathyrella candolleana* with Accession number - KF414680 and *Myrothecium gramineum* with Accession number - KF414681. The bootstrapped unrooted tree was structured by the neighbour joining method from the distance data generated by alignment of the nucleotide sequences. The phylogenetic tree generated from the 18S rDNA sequences of 11 taxa show the relationship of the 2 cultures with the reference taxa. The culture LCJ 177 shows a similarity with *Myrothecium gramineum* with 100 % bootstrap support (Fig. 2) and LCJ 178 with *Psathyrella candolleana* with 98% bootstrap support (Fig. 1). Only few reports are available on the production of laccase by Myrothecium sp. (Mou *et al.,* 1992). Zhao *et al.* (2012) reported the production of laccase by *Myrothecium verrucaria* and its ability to decolourize dyes. But this is the first report on laccase production by *Myrothecium gramineum*. Similar studies of isolation of laccase producing fungi were conducted by Priyadarshini *et al*. (2011). The isolated fungi was identified as *Trametes hirsute* based on the sequence analysis of the ITS region. Foorootanfer *et al.* (2011) also conducted a similar study and isolated a laccase producing ascomycete *Paraconiothyrium variabile.*

Conclusion

The present study showed the molecular identification of two fungal isolates *Psathyrella candolleana* LCJ 178 and *Myrothecium gramineum* LCJ 177 which have not been reported earlier for dye decolourization. The molecular techniques of identification are more significant than

morphological methods. Both the isolates exhibited laccase activity which can be used for decolourization and bioremediation studies. The laccase activity of the cultures can be enhanced further by optimization of the culture conditions.

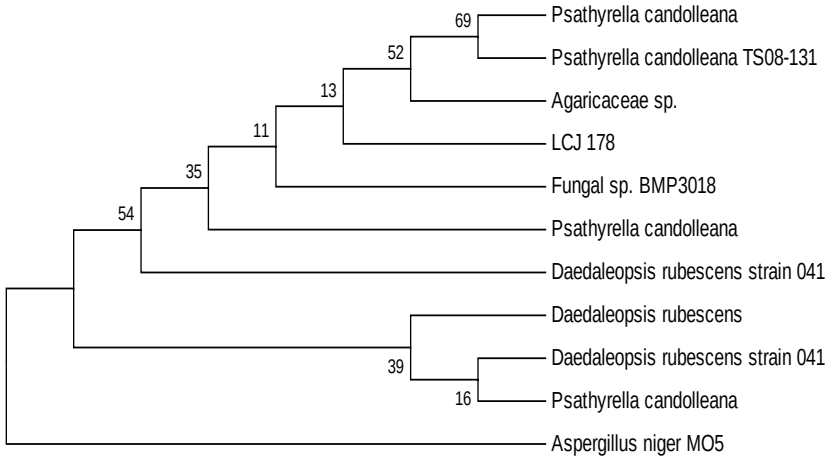

Figure 1: Phylogenetic tree of *Psathyrella candolleana* LCJ 178

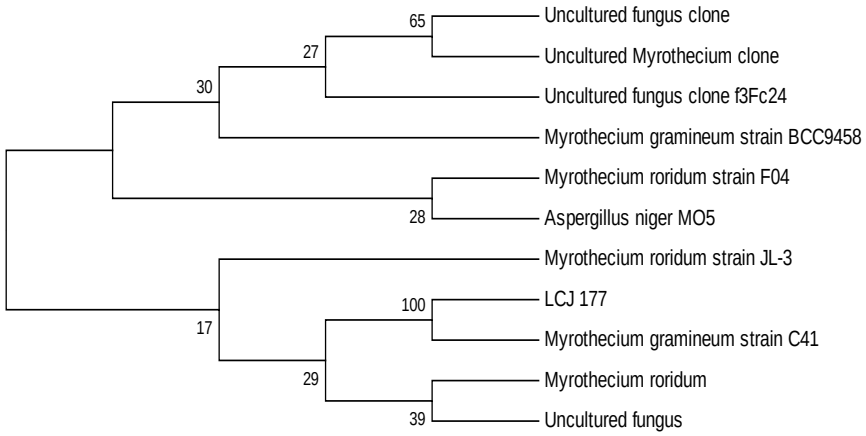

Figure 2: Phylogenetic tree of *Myrothecium gramineum* LCJ 177

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