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SCREENING AND MOLECULAR CHARACTERIZATION OF WHITE ROT FUNGI CAPABLE OF LACCASE PRODUCTION AND DYE DECOLOURIZATION

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ABSTRACT

This study describes the isolation of white rot fungi, their molecular characterization and screening for their ability to produce laccase and decolourize the synthetic dyes. Twenty two white rot fungi were isolated from the collected fruiting bodies and screened for their ability to produce laccase. Of these, ten isolates were laccase producers. Among the laccase producing fungi, three isolates namely LCJ155, LCJ164 and LCJ169 were also efficient in synthetic dye decolourization. The three promising isolates were identified by conventional and molecular methods. Fungal DNA was extracted, ITS-rDNA amplified by PCR, and ITS regions were sequenced. Based on the molecular characterization, the novel laccase producing isolates was identified as *Pleurotus floridanus* LCJ155 (Accession no. KF373566), *Leucocoprinus cretaceus* LCJ164 (Accession no. KF383135) and *Agaricus* sp. LCJ169 (Accession no. KF383136). The three isolates LCJ155, LCJ164 and LCJ169 can therefore be used in developing an eco-friendly technology for bioremediation of the dye containing effluents from dyestuff industries.

Keywords: White rot fungi, laccase, dye decolourization, rDNA-ITS sequences

1. INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2), are multicopper and lignin modifying enzyme (LME) found in bacteria, fungi, plants and insects. Among these, fungal laccase have accounted for the most important group of multicopper oxidase, especially basidiomycetous fungi (Bourbonnais et al. 1995; Palmieri et al. 2000). The isolation and identification of white rot fungi plays a vital role in exploiting its potential environmental and biotechnological application and to contribute to diversity description and conservation (Lee et al. 2006; Brock et al. 2009). Since 1990s, DNA-based techniques have been used for identifying white rot fungi (Glaeser and Lindner, 2010). For identification of specific genera and species, the rDNA repeat unit, consisting of the subunits 18, 5.8, 28, and 5S rDNA interrupted by the internal transcribed spacer (ITS) and the intergenic spacer (IGS) are employed due to their specific sequences as target regions (Schmidt et al. 2012). Though a number of DNA based identification methods are available, the specific advantage of ITS sequencing is to identify any fungal isolate and provided that the databases contain the corresponding sequence and that the sequence correlates with the correct fungal name (Schmidt et al. 2012). Laccase enable white rot fungi to degrade a wide range of pollutants which includes polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), pesticides, explosives, synthetic polymers and synthetic dyes (Pointing, 2001). Among these, synthetic dye containing effluents causes negative impact to the environment and they are toxic, mutagenic and carcinogenic to living being (Lee et al. 1999). Recently, research on biological treatment has offered a simple and cost effective ways of treating dye containing effluents. White

rot fungi have been studied for decades and new species are shown to decolourize a variety of dyes with their lignin degrading enzymes (Champagne and Ramsay, 2005; Gnanasalomi and Gnanadoss, 2013a; Jebapriya and Gnanadoss, 2013). The objectives of the present work was to isolate laccase producing white rot fungi from natural habitats, to screen these laccase positive isolates for synthetic dye decolourization and to identify potential laccase producing white rot fungal strains that are able to decolourize dyes using molecular and phylogentic analysis.

2. MATERIALS AND METHODS

2.1. COLLECTION AND ISOLATION OF FUNGI

Twenty two different fungal fruiting bodies (LCJ155-176) were collected from the tree trunks found in various parts of Chennai, India. The fruiting bodies were isolated from the soil, 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 75% ethanol for a few seconds under aseptic conditions and thoroughly washed with sterilized water. Fragments of basidiocarp were then inoculated on potato dextrose agar (PDA) plate and then incubated at room temperature. The cultures were purified by repeated transfer to fresh PDA plate and the pure cultures of the fungi were preserved on PDA slant culture at 4 °C.

2.2. SCREENING FOR LACCASE ACTIVITY

Primary screening of the isolates for laccase production was done on PDA plates supplemented with 4.0 mM guaiacol and 1.0 mM ABTS (2, 2´-Azino-bis-[3-ethylbenzthiazoline-6-sulphonic acid]) as a substrate. Twenty two isolates were inoculated on PDA plates containing the respective substrates and were incubated at room temperature. The production of an intense brown and dark green colour, under and around the fungal colony grown on PDA plates containing guaiacol and ABTS respectively was considered as a positive reaction for the presence of laccase activity. Fungal isolates showing positive reactions in the primary screening were subjected to secondary screening by culturing them in a medium containing glucose - 1 g, KH_2PO_4 - 0.1 g, $MgSO_4$ - 0.05 g, $CaCl_2$ - 0.014 g, yeast extract - 0.1 g, thiamine - 0.00025 g and distilled water 100 mL (Jonathan and Fasidi, 2001). Seven days old mycelial discs were inoculated and incubated at room temperature for 12 days on a shaker at 100 rpm. The culture was harvested on alternative days and centrifuged at 10,000 rpm for 15 min, in order to remove the mycelium and the clear supernatant containing extracellular enzymes was used for laccase assay. Laccase activity was assayed using 3 mL of 10 mM guaiacol in 100 mM acetate buffer (pH 5.0) containing 10% (v/v) acetone and 1 mL culture filtrate was added and then incubated for 15 minutes. The enzymatic activity was assayed by measuring oxidation of guaiacol at 470 nm (Collins and Dobson, 1997). Laccase activity was calculated according to the method described by Eichlerova et al. (2012). One unit of enzyme activity was defined as the amount of enzymes that oxidizes 1µmol of guaiacol.

2.3. SCREENING FOR DYE DECOLOURIZATION

The decolourization experiment was carried out by using the method described by Radhika et al. (2013). Under qualitative screening, PDA plates were supplemented with Bromophenol blue, Brilliant green, Phenol red, Congo red and Methyl red at 0.05% (w/v) respectively. The dye containing PDA plates were then inoculated with mycelial discs obtained from actively growing culture on PDA plate. The decolourization of dyes was observed by the formation of decolourization zones under and around developing mycelia.

Quantitative decolourization studies were carried using cultures grown in 250 mL Erlenmeyer flasks containing 100 mL of basal medium Jonathan and Fasidi, 2001 (g/L): Glucose -10, KH_2PO_4 - 1, $MgSO_4$ - 0.5, $CaCl_2$ - 0.14, Yeast extract - 1, Thiamine - 0.0025. 0.05% of the respective dyes were added to the medium. The culture medium containing the respective dyes was autoclaved at 121°C for 15 minutes. Three fungal mycelial discs (6 mm) were inoculated into each conical flask under sterile condition and incubated on the rotary shaker maintained at 120 rpm under room temperature. The OD of each of the sample was taken at the respective absorption maxima of each of the dye at different time intervals. The percentage of decolourization was calculated using the following equation:

$$\text{Decolourization (\%)} = \frac{(I - F)}{(F)} \times 100$$

Where
I = Initial absorbance
F = Final absorbance

2.4. MOLECULAR CHARACTERIZATION OF FUNGI

Comparison of the internal transcribed spacer (ITS) ribosomal DNA (rDNA) gene sequence was employed for molecular characterization. Genomic DNA of the isolated strain was extracted with universal genomic DNA extraction kit (HELINI biomolecules). The gene encoding ITS region was amplified by the fungi identification polymerase chain reaction (PCR) kit. After purification of the amplified products by agarose gel DNA purification kit, ITS rDNA gene was sequenced in both directions with ITS 1 and ITS 4 primers (White et al. 1990) at Solgent Co., Ltd., Korea. Sequences generated were then assembled into one complete sequence using Bioedit (Hall, 1999) and the most homologous sequences were determined in comparison to the GenBank database using BLAST (Altschul et al. 1997). Finally, genetic distances and neighbor-joining algorithm were analyzed using MEGA 5.

3. RESULTS AND DISCUSSION

3.1. SCREENING OF LACCASE PRODUCING FUNGI

Most of the white rot fungi produce laccases (Eriksson et al. 1990). In the present investigation, twenty two basidiomycetous fungi were screened for laccase activity using the chromogenic screening method on PDA-guaiacol medium. Table 1 summarizes the results of primary screening for the laccase producing fungal isolates. Of the twenty two isolates, ten isolates were laccase positive. On performing qualitative screening by using ABTS and guaiacol as a substrate the fungal isolates formed deep green and brown zone respectively around and above the colony which is a positive confirmation for laccase activity. Similarly, Arora and Sandhu (1985) used lignin guaiacol agar medium for screening such fungi and Kiiskinen et al. (2004) cultivated fungi on agar media containing indicator compounds (such as RBBR, poly R-478, guaiacol, and tannic acid). Apart from this, indicators like 2, 2´-Azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) is also suitable for screening because its one electron oxidation product is soluble in water, stable, and intensely green. Field et al. (1992) demonstrated that a polymeric dye could be used for screening white rot fungi. After primary screening, cultures were subjected to secondary screening. The isolates LCJ155, LCJ164 and LCJ169 were found to produce high activities of laccase during submerged fermentation. Quantitative experiments in shake flask culture showed a laccase activity of 0.807, 0.777 and 0.668 U/mL on the eighth day (Figure 1). Hence, cultures LCJ155, LCJ164 and LCJ169 were selected for further investigation.

Table 1
Primary screening of the isolated fungal strains

Isolates No.	Habitat	ABTs oxidation	Guaiacol oxidation
LCJ155	Tree trunk	++++	++++
LCJ 156	Decayed wood	-	-
LCJ 157	Soil	-	-
LCJ 158	Soil	-	-
LCJ 159	Soil	++	++
LCJ 160	Soil	-	-
LCJ 161	Tree trunk	-	-
LCJ 162	Soil	-	-
LCJ 163	Soil	-	-
LCJ164	Tree trunk	++++	++++
LCJ 165	Decayed wood	++	++
LCJ 166	Soil	-	-
LCJ 167	Decayed wood	-	-
LCJ 168	Soil	-	-
LCJ169	Tree trunk	+++	++++
LCJ 170	Tree trunk	+	++
LCJ 171	Tree trunk	++	++
LCJ 172	Soil	-	-
LCJ 173	Tree trunk	+++	+++
LCJ 174	Decayed wood	-	-
LCJ 175	Soil	++++	+++
LCJ 176	Tree trunk	++	++

(Oxidation scale: + 0-1 cm; ++ 1-3 cm; +++ 3-4 cm; 4-5 cm; - absent)

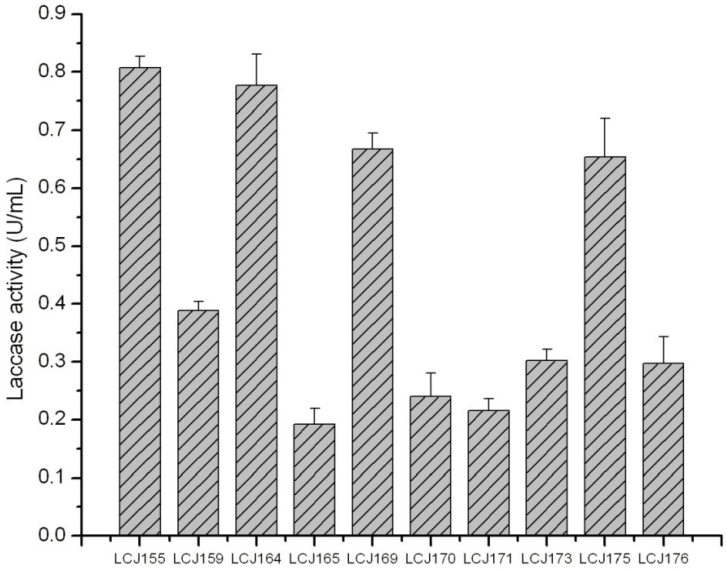

Figure 1
Secondary screening for laccase production by the different isolates

3.2. SYNTHETIC DYE DECOLOURIZATION USING LACCASE POSITIVE FUNGI

Certain fungal laccase are efficient in dye decolourization (Kirby et al. 2000; Chagas and Durrant, 2001; Champagne and Ramsay, 2005; Harazono and Nakamura, 2005). In the present study, laccase positive isolates were investigated for their ability to decolourize five synthetic dyes namely brilliant green, bromophenol blue, congo red, methyl red and phenol red by cultivation on a solid media. The decolourization abilities of the fungal strains after 15 days of incubation are shown in Table 2. Among the five dyes tested in this study, brilliant green, congo red and methyl red dyes were far the most rapidly decolourized, followed by the bromophenol and phenol red. LCJ 156, 157, 158, 160, 161, 162, 163, 165, 166, 167 and 168 were not able to decolourize all three dyes. Only slow and incomplete decolourization was observed. In contrast, LCJ155, 164, and 169 were able to completely decolourize all three dyes within 10 days. Decolourization was observed by the formation of clear zones with biosorption around the colonies, and complete decolourization was assessed as the total disappearance of the colour without visible biosorption to the biomass. A number of studies have reported that white rot fungi have the ability to degrade synthetic dyes (Harazono and Nakamura, 2005). The data obtained in the present study with laccase positive fungal isolates support the observations of Selvam et al. (2003) who isolated white rot fungi for decolourization of azo dyes. Sathiyamoorthi et al. (2007) tested the white rot fungi *Trametes hirsute* and *P. florida* for their dye decolourizing ability against reactive dyes Blue CA, Black B133 and Corazol violet SR. Similarly, Saritha et al. (2010) and Radhika et al. (2013) employed the method of plate decolourization studies using white rot fungi.

Table 2
Qualitative screening for dye decolourization using laccase positive isolates using agar plates

Isolates No.	Decolourization zone				
	Brilliant Green	Bromophenol Blue	Congo Red	Methyl Red	Phenol Red
LCJ155	+	+	++	++++	-
LCJ 159	++	+	+	++	-
LCJ164	+++	++	++++	++++	+
LCJ 165	++	++	++	+	+
LCJ169	+++	++	+++	++++	+
LCJ 170	+	+	+++	+++	-
LCJ 171	++	+	++	++	+
LCJ 173	++++	+	+++	++++	-
LCJ 175	++++	+++	++++	++++	++
LCJ 176	+	+	-	+	-

(Decolourization zone: ++++ completely decolourized; +++ high; ++ moderate; + less and - negative)

The decolourization ability (quantitative) of laccase positive isolates was carried out in basal medium. Decolourization percentage of the fungal isolates is presented in Table 3. Under experimental conditions, each dye showed a different decolourization tendency, brilliant green, congo red and methyl red were decolourized faster than bromophenol blue and phenol red. Results showed that the following isolates have the best decolourizing capabilities: LCJ155 (brilliant green 84%, bromophenol blue 47%, congo red 81%, methyl red 82% and phenol red 46%), LCJ164 (brilliant green 98%, bromophenol blue 49%, congo red 93%, methyl red 91% and phenol red 37%) and LCJ169 (brilliant green 77%, bromophenol blue 51%, congo red 78%, methyl red 88% and phenol red 63%). The ability of white rot fungi to decolourize various synthetic dyes has been extensively studied (Jarosz et al. 2002; Champagne and Ramsay, 2005). In view of existing literature regarding dye decolourization by white rot fungi, lignin modified enzymes proves its extensive role in dye decolourization through its mechanism of mineralization of dyes (Jebapriya and Gnanadoss, 2013). In recent years, white rot fungal laccase has been well studied in relation to dye degradation which includes *Phlebia tremellose* (Kirby et al. 2000), *Pleurotus sajorcaju* (Chagas and Durrant, 2001), *Trametes hirsute*, *T. modesta*, *Sclerotium roysii* (Nyanhongo et al. 2002) *Laccaria fraterna, P. ostreatus* (Balaraju et al. 2007; Gnanadoss et al. 2013) and *Psathyrella candolleana* LCJ178 (Gnanasalomi and Gnanadoss, 2013b).

Table 3

Quantitative screening for dye decolourization using laccase positive isolates using dye containing medium

Isolates No.	% Decolourization				
	Brilliant green	Bromophenol blue	Congo red	Methyl red	Phenol red
LCJ155	82	47	81	82	46
LCJ 159	98	49	93	91	37
LCJ164	77	51	78	88	63
LCJ 165	52	43	36	48	10
LCJ169	70	10	65	25	40
LCJ 170	57	12	39	43	11
LCJ 171	76	22	84	70	36
LCJ 173	61	21	90	73	27
LCJ 175	64	46	85	72	07
LCJ 176	33	14	44	38	28

3.3. MOLECULAR CHARACTERIZATION OF LACCASE POSITIVE FUNGI

The morphological characteristics of three promising laccase producing and dye decolourizing fungal strains (LCJ155, LCJ164 and LCJ169) were observed. As the morphological characteristics were not promising, 18S rDNA sequencing was used for the identification. The ITS region of isolates LCJ155, LCJ164 and LCJ169 was amplified, sequenced and submitted to GenBank (Accession no. KF373566, KF383135 and KF383136). The obtained sequence was compared with those in the National Center for Biotechnology Information nucleotide Sequence database by using the basic local alignment search tool (BLAST) algorithm. LCJ155 18S rDNA (Accession no. KF373566) showed 99% homology with *Pleurotus floridanus* (Accession no JQ868739.1), LCJ164 18S rDNA (Accession no. KF383135) showed 99% homology with *Leucocoprinus cretaceus* (Accession no AF482861.1) and LCJ169 18S rDNA (Accession no. KF383136) showed 97% homology with *Agaricus* sp. (Accession no JF727861.1). The phenograms reflecting the phylogenetic relationship between LCJ155, LCJ164 and LCJ169 constructed using data from the BLAST analysis of the rDNA region of LCJ155, LCJ164 and LCJ169 are shown separately in Figure 2, 3 and 4. The phylogenetic relationship of LCJ155, LCJ164 and LCJ169 was inferred using the Neighbor-Joining method. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5. Based on the analysis of ITS rDNA gene sequence, the isolates LCJ155, LCJ164 and LCJ169 were identified as *Pleurotus floridanus*, *Leucocoprinus cretaceus* and *Agaricus* sp. respectively and named as *Pleurotus floridanus* LCJ155, *Leucocoprinus cretaceus* LCJ164 and *Agaricus* sp. LCJ169.

Yang et al. (2009) isolated a white rot fungus, strain SQ01 from decayed wood in a temperate forest and identified as a member of genus *Trametes*, based on the morphological

characteristics and a complete sequence analysis of its 18S rRNA gene and ITS region. The internal transcribed spacer (ITS) region of fungal rDNA has been successfully used for species identification. The ITS region is present at a very high copy number in the genome of fungi, as part of tandem repeated nuclear rDNA which coupled with PCR amplification produces a highly sensitive assay (Jasalvich et al. 2000). Nicolotti et al. (2009) identified decayed wood fungal samples by amplified ITS region with the primers pair of ITS1 and ITS4. Many primer sets are designed to target the conserved regions of fungal rRNA (Turenne et al. 1999) increasing the sensitivity and selectivity for species identification. The earliest PCR primer sets to be routinely used to amplify the fungal ITS regions were the fungal specific primer ITS1 and the fungal general primer ITS4 (White et al. 1990). Numerous studies have used PCR-based methods to identify fungi, but the specific primers and the number of isolates per species used in each study have varied (Diehl et al. 2004).

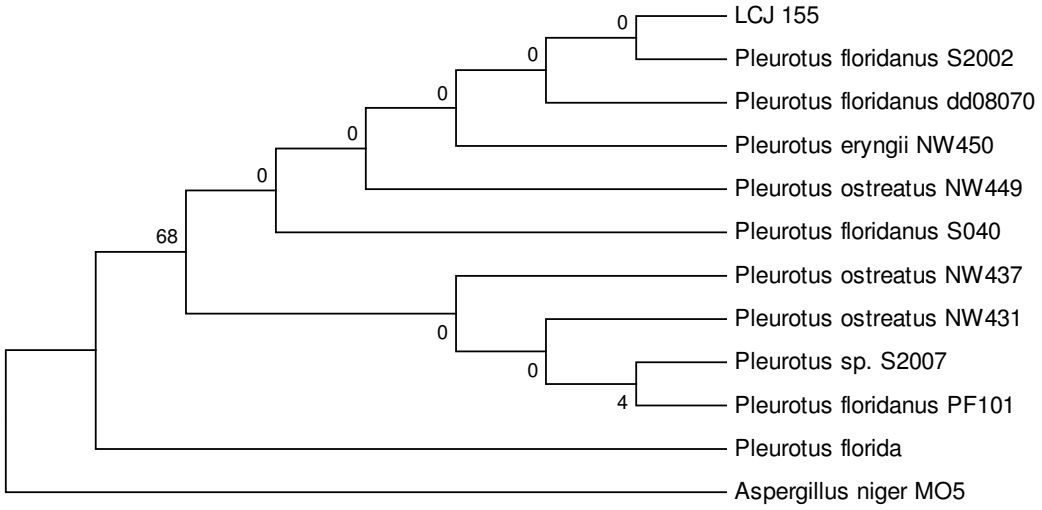

Figure 2
Neighbor-Joining tree of the ITS region of strains LCJ155 and with higher similarity from the genbank selected from the results of a BLASTn search

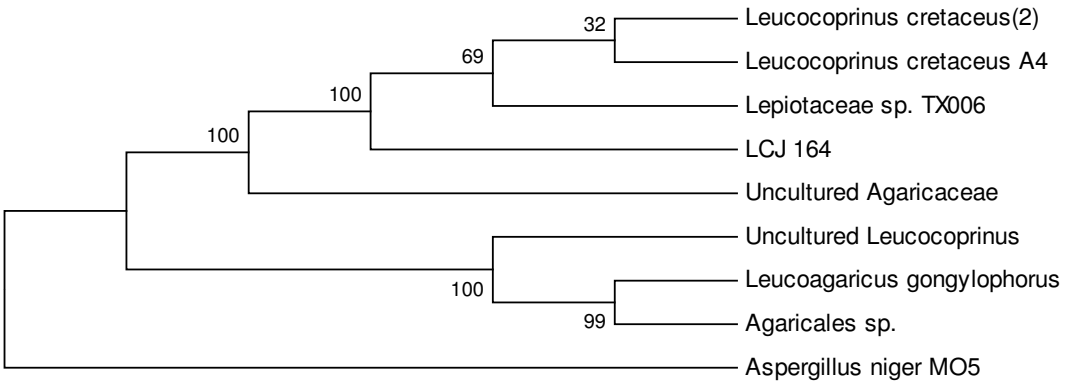

Figure 3
Neighbor-Joining tree of the ITS region of strains LCJ164 and with higher similarity from the genbank selected from the results of a BLASTn search

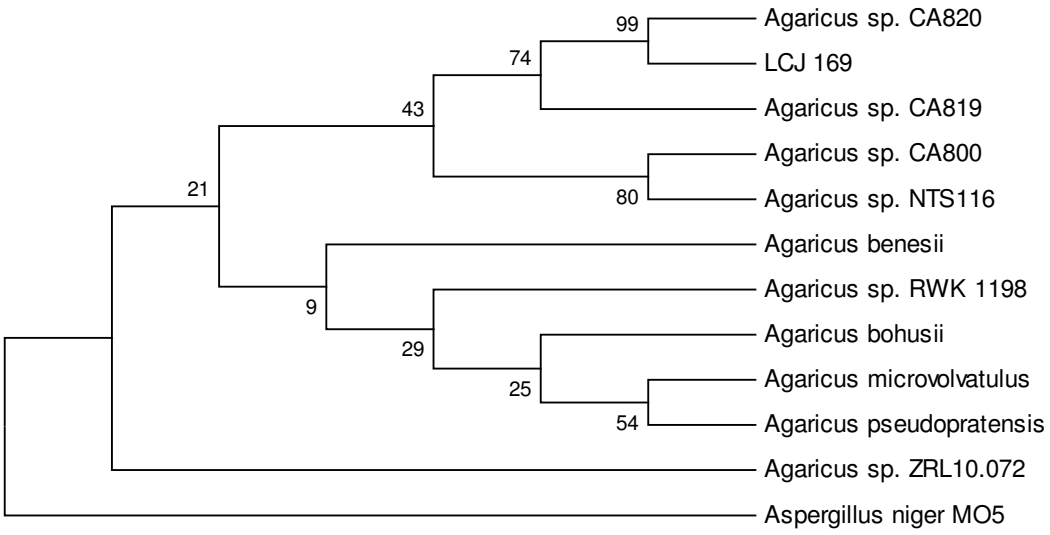

Figure 4
Neighbor-Joining tree of the ITS region of strains LCJ169 and with higher similarity from the genbank selected from the results of a BLASTn search

The present study highlights the exploitation of laccase producing white rot fungi as an alternative method for treatments of dye containing effluents. Treatment of coloured effluents using laccase from white rot fungi will prove to be environmentally friendly and cost-effective. Optimizing the condition for increasing laccase production and decolourization of dyes needs further investigation.

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