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Characterization and Identification of Potent LMEs (Lignin Modifying Enzymes) Producing Fungi from Dang (Gujarat)

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Abstract

Characterization and identification of potent LMEs (Lignin Modifying Enzymes) producing fungi from Dang (Gujarat) was conducted at Department of Plant Pathology, College of Agriculture, Navsari Agricultural University, Waghai in 2021-22, Fungal isolates LD1, LD4, LD5, LD9, LD15, LD19, LD20 and LD39 were screened for Lignin Modifying Enzymes (LME) on ABTS (2, 2 azinobis (3-ethylbenz-thiazoline)-6 sulphonate), Methyl orange and α- naphthol agar and observed that all the fungal strains showed color zone/clear zone on different indicator plates. However, fungal strain LD19 showed the highest potency index on both ABTS and methyl orange plates. Among the 8 selected fungal isolates, 5 produced all the three enzymes *viz.* laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP), 2 produced two enzymes while 1 produced only one enzyme. Isolate LD19 had given maximum laccase activity at all the intervals and it was maximum at 6 DAI (206.74 U ml^{-1}) followed by LD20 (144.79 U ml^{-1}), LD4 (118.38 U ml^{-1}) and LD15 (113.68 U ml^{-1}). Maximum LiP activity was recorded in LD19 (1.314 U ml^{-1}) at 8 DAI followed by LD1 (0.903 U ml^{-1}) and it was lowest in LD39 (0.725 U ml^{-1}). Maximum MnP activity was recorded in LD19 (428.65 U ml^{-1}) at 8 DAI followed by LD39 (308.13 U ml^{-1}), LD15 (290.18 U ml^{-1}), LD4 (262.16 U ml^{-1}) and MnP activity was not detected in LD1 and LD5. The highest LMEs were observed in LD19. Potential LMEs producing fungi LD19 wasidentified as *Schizophyllum commune,* by morphological, microscopic characteristics and molecular methods and submitted to NCBI GenBank database with accession numbers MW720154.

Keywords: ABTS, Methyl orange, α- naphthol, *Schizophyllum commune*

1. Introduction

Lignin is the most structurally complex carbohydrate possessing a high molecular weight and the most recalcitrant, consisting of various biologically stable linkages (Perez et al., 2001). The lignocellulose material of plant consists of three main compounds, namely cellulose, hemicellulose and lignin. After cellulose, lignin is the second most abundant renewable biopolymer in nature. It is most abundant aromatic polymer in the biosphere (Rahmanet al., 2013). Lignin, the second-most abundant biopolymer on Earth and a heterogeneous polymer in lignocellulosic residues, is the only naturally synthesised polymer with an aromatic backbone. It generally contains three precursor aromatic alcohols including coniferyl alcohol, sinapyl and p-coumaryl(Weiet al.,2009). These precursors form the guaiacyl- (G), syringyl- (S) and p-hydroxyphenyl (H) subunits in the lignin molecule, respectively (Martinezet al., 2005). The subunits ratio, and consequently, the lignin composition, varies between different plant groups. Oxidative coupling of these lignin aromatic alcohol monomers creates a complex structure in lignin which is highly recalcitrant to degradation (Wong, 2009). Lignin causes a serious pollution and toxicity problem in aquatic ecosystem owing to its low biodegradability. Large amounts of lignocellulosic waste generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro-industries pose environmental pollution problems (Howard et al., 2003).

Ligninolytic microorganisms play a major role in recycling the abundant biomass resources on earth through biodegradationof lignin.Ligninolytic microorganisms are equipped with various enzymes which can degrade lignin. Many bacteria, actinomycetesand fungi are reported to produce Lignin Modifying Enzymes (LMEs) to degrade lignin components of agro-waste. Through the synergistic action of microorganisms, complex organic compounds are degraded to

smaller molecules, which can then be utilized by microbial cells (Golueke (1991). The degradation of lignin is brought about by fungi mainly belonging to Basidiomycetes (Rao, 2008). Over 600 species of Basidiomycetes have been found to be ligninolytic converting lignin to CO_2, by secreting extracellular enzymes (Kumar and Gupta, 2006). Basidiomycetes are well known for their lignin degrading enzymes, commonly laccase and the perioxidases such as lignin peroxidase (LiP) and manganese peroxidae (MnP) (Srinivasan et al., 1995). In addition, the following enzymes are also associated with lignin breakdown, but are unable to degrade lignin alone: glyoxal oxidase, superoxide dismutase, glucose oxidase, aryl-alcohol oxidase and cellobiose dehydrogenase. They produce the H_2O_2 required by peroxidases (LiP and MnP) or serve to link lignocellulose degradation pathways (Hofrichter, 2002; Hernandez-Ortega et al., 2012). In many cases, the advantage of fungal enzymes over bacterial ones for biodegradation of organopollutants is attributed to their extracellular nature, which also makes them suitable for degradation of various aromatic compounds, i.e. substrates with low water solubility. In addition, the fungi produce low molecular weight mediators that extend the spectrum of pollutants they are able to oxidize (Pointing, 2001; Majeau et al., 2010; Cajthaml and Svobodová, 2012). The important lignin degrading fungi are *Clavaria, Clitocybecollybia, Flammula, Hypholoma, Lepiota, Mycena, Pleurotus, Agaricus, Polyporus, Fusarium, Arthrobotrys, Poria, Pholiota, Cephalosporium, Collybi,* and *Humicola* (Atlas and Bartha, 1998).

Lignin degading enzymes lignin peroxidase and laccase produced by *Schizophyllum commune* Vijya and Reddy (2012). Kumar et al. (2018) and Aswal et al. (2020) also reported *Schizophyllum commune* as lignin degrading fungi. Ligninolytic fungi *Schizophyllum commune* were identified by macroscopic, microscopic and molecular methods Singh et al. (2017) and Kumar et al. (2018). In this study, an attempt was made to screen ligninolytic potentials of lignin degrading fungi on the basis potency index values by using ABTS, Methyl orange and α- naphtholas screening reagents as well as characterization and identification of potent ligninolytic fungi from The Dangs (Gujarat).

2. Materials and Methods

Characterization and identification of potent LMEs (Lignin Modifying Enzymes) producing fungi from Dang (Gujarat) was conducted at Department of Plant Pathology, College of Agriculture, Navsari Agricultural University, Waghai in 2021–22.

2.1. Microorganism and culture condition

A total 8 out of 43 previously isolated ligninolytic fungi (LD1, LD4, LD5, LD9, LD15, LD19, LD20 and LD39) from the different locations of the Dangs (Gujarat) were selected based on primary screening for the study.The culture was maintained on potato dextrose agar at 4^0C and sub-cultured regularly.

2.2. Screening of Ligninolytic fungi through various agar

Ligninolytic fungal isolates LD1, LD4, LD5, LD9, LD15, LD19, LD20 and LD39 were inoculated on ABTS agar (LBM supplemented with 0.1% w/v ABTS and 1 ml of 20% w/v aqueous glucose (separately sterilized)), Methyl orange agar (PDA supplemented with 0.5% w/v methyl orange) and α-naphthol agar (LBM supplemented with 0.005% w/v α-naphthol and 1 ml of 20% w/v aqueous glucose) for comparison of potency index. Inoculated plates were incubated at 26±2˚C in the incubator for 5 days and observed for clearance or color zone produced around the mycelium/ colony. The potency index (PI) was calculated using the following equation (equation (1)) (Tecket al. (2011).

2.3. Quantitative screening of lignin modifying enzyme (LME) activity of fungi

For quantification of Lignin Modifying Enzyme (LME), 5 mm disc of fungal isolates were inoculated separately into 100 ml of MSM broth supplemented with 1% alkaline lignin. Flasks were incubated at 26±2˚C in rotary shaker up to 10 days. Samples were withdraw and filter at 2 days interval and filtrate was collected and used as crude enzyme sample for laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP) enzymes.

2.3.1. Laccase

Laccase activity was measured by monitoring the oxidation of 2, 2 azinobis (3-ethylbenz-thiazoline)-6 sulphonate (ABTS) from the crude enzyme (Wolfenden and Wilson (1982)) sample at pH 4.5 and 35˚C temperature. Laccase activity assay was performed in a 2.1 ml reaction mixture containing 1 ml of 0.1 M sodium acetate buffer (pH 4.5), 1 ml of 1 mM ABTS and 0.1 ml of enzyme solution, and ABTS oxidation was followed at 420 nm (ε_{420} 36000 M cm^{-1}).

2.3.2. Lignin peroxidase (LiP)

The LiP was estimated using demethylation of the methylene blue dye and measured on a spectrophotometer (Denise et al. (1996)). In presence of H_2O_2, methylene blue is converted in Azure C a leuco compound by LiP. The assay mixture of 3.0 ml contains 2.1 ml of distilled water, 0.1 ml of 1.2 mM methylene blue, 0.6 ml of 0.5 M sodium tartrate buffer (pH 4.0) and 0.1 ml of the enzyme. The reaction was started by the addition of 0.1 ml of 2.7 mM H_2O_2 as an inducer. The conversion of the dye to Azure C was monitored by the measurement of the decrease in absorbance at 664 nm.

2.3.3. Manganese peroxidase (MnP)

MnP activity was estimated by monitoring the oxidation of Mn^{2+} to Mn^{3+} at 270 nm A_{270} (ε270, 11590 M^{-1} cm^{-1}) using a spectrophotometer, according to the method of Wariishiet al., (1992). The assay reaction mixture containing 0.4 ml 1.0 mM $MnSO_4$ in 1.0 ml 50.0 mM sodium malonate buffer (pH 4.5) and 0.1 ml of crude enzyme followed by incubation at 28˚C for 30 min. start the reaction by adding 0.4 ml 0.1 mM H_2O_2. Control was prepared similarly by the addition of

distilled water instead of enzyme sample. Immediately after adding H_2O_2 absorbance wasmeasured at 270 nm at room temperature.

2.4. Characterization and Identification of potential ligninolytic fungi

Potent ligninolytic fungal isolate was characterized in terms of macroscopic characteristics (color, appearance, etc.) on Potato Dextrose Agar (PDA) and microscopic characteristics (hyphae, clamp connections, spicules, spores, etc.) by microscopic examination.Most potential ligninolytic fungi were identified using ITS based primer (Petti(2007)).For the identification of ligninolytic fungi, DNA was extracted from the culture by the CTAB method and its quality was evaluated on 1.0% agarose gel. Fragment of ITS gene was amplified using ITS primer by PCR followed by purification to remove contamination in column purification system. DNA sequencing was done at Saffron Life Sciences, Surat. Briefly, sequencing was carried out with ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') primer using BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic Analyzer. Quarry sequence was searched for homology on NCBI using the BLAST tool. Based on maximum identity score and homology with available sequences, a phylogenetic tree was constructed using NCBI web tools and the sequence was submitted to NCB1-GeneBank to obtain accession number.

3. Results and Discussion

3.1. Screening of ligninolytic fungi through various agar

Ligninolytic fungal isolates LD1, LD4, LD5, LD9, LD15, LD19, LD20 and LD39 were compared for ligninolytic potential on different indicators plate *viz.* ABTS agar, methyl orange agar and α-naphthol agar (Table 1). All the fungal strains produced green color around the colony in ABTS agar which indicates the presence of laccase enzyme. A significantly higher potency index was observed in isolate LD19 (2.32) on ABTS agar, next in order of potency was in LD20 (1.90), followed by LD15 (1.63) which was statistically at par with LD4 (1.61), LD9 (1.58) and LD1 (1.52) whereas, lowest potency index was observed in LD5 (1.36) (Figure 1).

Table 1: Potency index and the reaction of ligninolytic fungi on different agar media

Sl. No.	Strain No.	ABTS agar	Methyl Orange agar	α-Naphthol agar
		Potency index		Reaction
1	LD1	1.52c	1.24d	+
2	LD4	1.61c	1.33bc	+
3	LD5	1.36d	1.28cd	-
4	LD9	1.58c	1.28cd	+
5	LD15	1.63c	1.37b	+
6	LD19	2.32a	1.42a	+
7	LD20	1.90b	1.36b	+
8	LD39	1.54c	1.27d	+
SEm ±		0.03	0.02	
CD (*p*=0.05)		0.10	0.05	
CV %		3.55	2.12	

Note: Different letters in the same column indicate significant differences

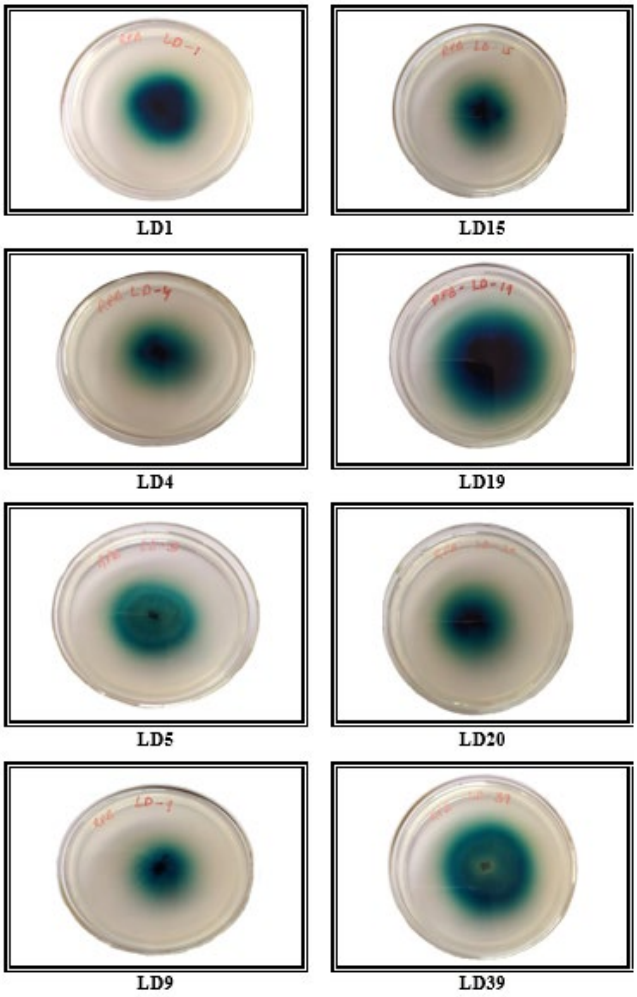

Figure 1: Zone of lignin degradation by ligninolytic fungi on ABTS agar

Isolate LD19 showed a significantly higher potency index (1.42) on methyl orange plate, it was followed by LD15 (1.37) which was statistically at par with LD20 (1.36) and LD4 (1.33) whilst, the lowest potency index was observed in isolate LD1 (1.24) which was at par with LD5 (1.28) and LD9 (1.28) (Figure 2). Amongst 8 selected fungal strains, 7 fungal strains except LD5 produced dark violet color on α- naphthol agar indicated the presence of laccase (Figure 3).

Overall, it was observed that all the selected fungal strain showed color zone/clear zone on different indicator plates and fungal strain LD19 showed the highest potency index on both ABTS and methyl orange plates. Different researchers have reported indicators ABTS, methyl orange and naphthol

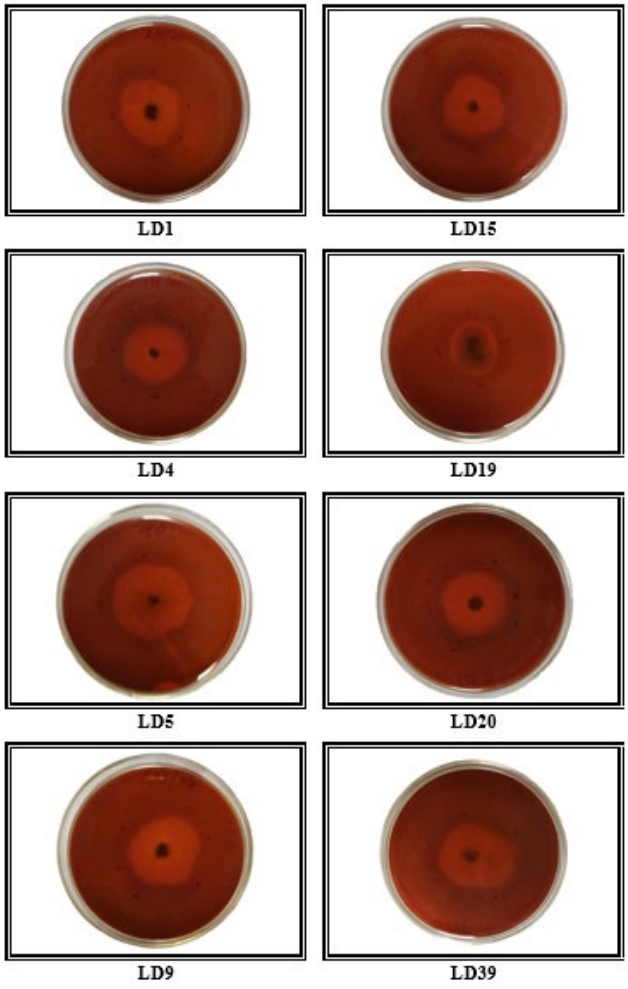

Figure 2: Zone of lignin degradation by ligninolytic fungi on methyl orange agar

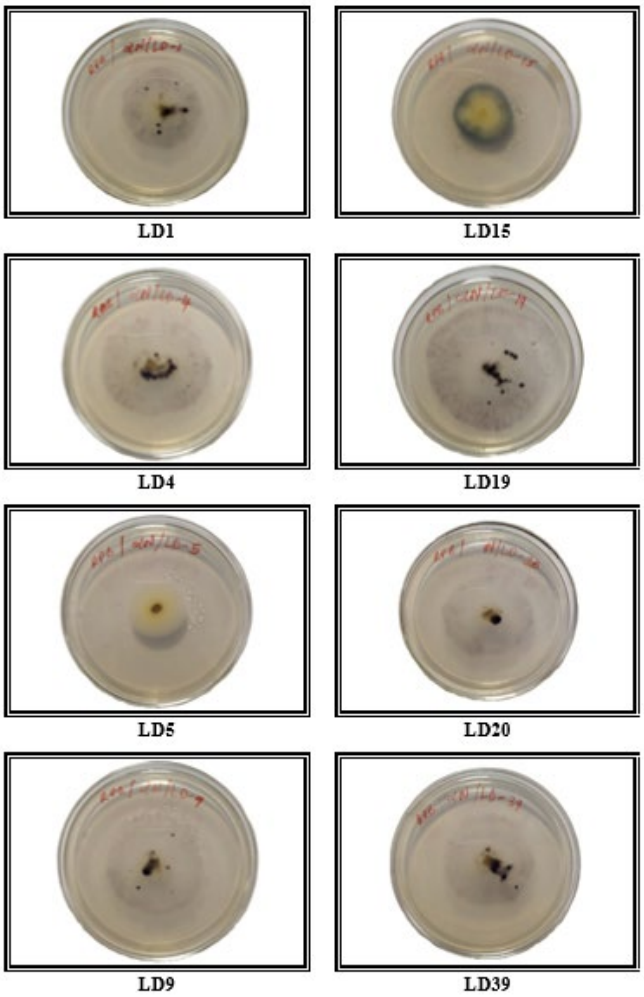

Figure 3: The reaction of ligninolytic fungi on α-naphthol agar

for the detection of lignin-modifying enzymes Dhouib et al., (2005), Sharma et al. (2017) and Rajwar et al. (2016) respectively. Kauret al. (2018) reported that 0.08% ABTS gave better visual expressions as well as potency index among the other chromogenic indicators.

3.2. Quantitative screening of lignin modifying enzymes (LMEs) activity of fungi

Fungalstrains were further screened for quantitative production of Lignin Modifying Enzymes (LMEs) in MSM broth supplemented with 1% alkaline lignin. The major enzymes associated with lignin degradation are laccase, lignin peroxidase and manganese peroxidase.

Laccase activity of selected fungal strains was recorded up to 10 DAI at 2 days intervals (Figure 4). Significantly higher laccase activity was recorded in LD19 (59.93 U ml^{-1}) followed by LD20 (55.26 U ml^{-1}), LD4 (49.58 U ml^{-1}) which was statistically at par with LD9 (48.96 U ml^{-1}) whereas, the lowest laccase activity was recorded in LD5 (30.80 U ml^{-1}) at 2 DAI. Laccase activity of all the fungal strains was increased up to 6 DAI except LD5 and LD15 which was increased up to 8 DAI then after it

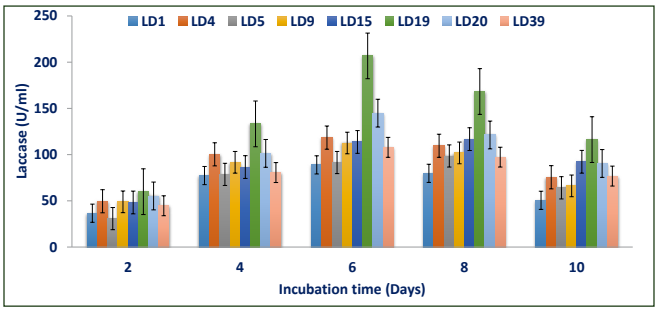

Figure 4: Laccase enzyme activity of ligninolytic fungi at different incubation periods

was decreased in all the fungal strain. Moreover, LD19 had given maximum laccase activity at all intervals. While, it was significantly maximum at 6 DAI 206.74 U ml^{-1} followed by LD20 (144.79 U ml^{-1}), LD4 (118.38 U ml^{-1}), LD15 (113.68 U ml^{-1}) which was at par with LD9 (112.59 U/ml) whilst, it was recorded lowest in LD1 (88.86 U ml^{-1}).

Lignin peroxidase (LiP) activity of isolates was recorded at 2 days intervals up to 10 days (Figure 5). Amongst the 8 selected isolates, LD19 had given significantly higher LiP activity at

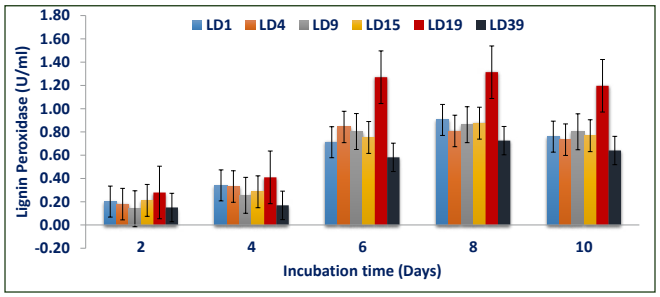

Figure 5: Lignin Peroxidase (LiP) enzyme activity of ligninolytic fungi at different incubation periods

all the intervals. However, LiP was not detected in LD5 and LD20 isolates. Maximum LiP activity was recorded in LD19 (1.314 U ml^{-1}) at 8 DAI followed by LD1 (0.903 U ml^{-1}) which was statistically at par with LD9 (0.862 U ml^{-1}) while, the lowest LiP was recorded in LD39 (0.725 U ml^{-1}). LiP activities of isolates LD1, LD9, LD15, LD19 and LD39 were increased up to 8 days and then after decreased from 10th day while, LiP activity of LD4 isolate was increased up to 6 days and then it was decreased.

Manganese peroxidase (MnP) activity of selected isolates is given in Figure 6. Significantly maximum MnP activity was recorded in LD19 (428.65 U ml^{-1}) on the 8th day followed by LD39 (308.13 U ml^{-1}), LD15 (290.18 U ml^{-1}), LD4 (262.16 U ml^{-1}) which was at par with LD20 (259.81 U ml^{-1}) whereas, lowest MnP activity was recorded in LD9 (234.55 U ml^{-1}). However, MnP activity was not detected in LD1 and LD5 amongst selected isolates. Ematouet al. (2020) reported that out of 21 isolated species, 18 produced all the three enzymes *viz.* laccase, lignin peroxidase and manganese peroxidase while 3 produced two enzymes. Laccase was found in all species and had the highest specific activity (0.0220 to 17.5994 U mg^{-1}) followed by manganese peroxidase (0.0005 to 0.1992 U mg^{-1}) and lignin peroxidase (0.0005 to 0.0278 U mg^{-1}). They have also reported that the highest ligninolytic enzyme content was found in *Coriolopsis polyzona* with specific activities of laccase, manganese peroxidase and lignin peroxidase of 17.5994 U mg *Coriolopsispolyzona*, 0.1336 U mg^{-1} and 0.0007 U mg^{-1}, respectively. Kathirgamanathan et al. (2017) also reported that among the eighteen basidiomycetes isolates *Earliella scabrosa* produced the highest laccase (91.2 U/l) and Mnperoxidase (17.5 U/l) activity. Moreover lignin peroxidase activity was not detected from the isolates.

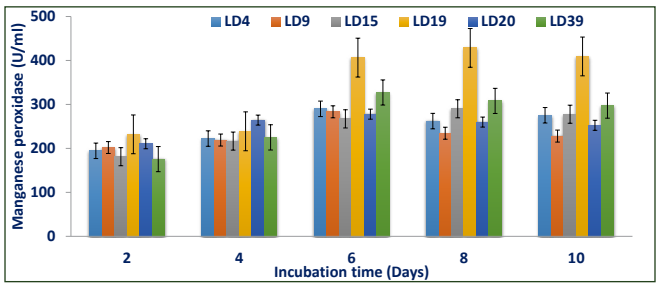

Figure 6: Manganese peroxidase (MnP) enzyme activity of ligninolytic fungi at different incubation periods

Irshad and Asgher (2011) reported that the maximum activity of enzymes MnP, LiP and laccase produced by *Schizophyllum commune* IBL-06 was 3745 IU ml^{-1}, 2700 IU ml^{-1} and 345 IU ml^{-1} respectively after 3 days of incubation in solid-state fermentation using banana stalk as substrate. Vijya and Reddy (2012) also reported that lignin peroxidase and laccase activity of *Schizophyllum commune* in malt extract broth amended with 20% paddy straw was 92 U ml^{-1} and 87 U ml^{-1}, respectively at 7th day of incubation then increase to 125 U ml^{-1} and 138 U ml^{-1} at 14th day. Further, it was increased to 164 U/ml LiP and 152 U ml^{-1} Lac.

Among the 8 selected isolates, 5 produced all the three enzymes *viz.* laccase, lignin peroxidase and manganese peroxidase, 2 produced two enzymes while 1 produced only one enzyme. The highest LMEs were observed in LD19. Hence, isolate LD19 was selected for further characterization and identification.

3.3. Characterization and identification of potent ligninolytic fungi

Potent ligninolytic isolate LD19 was characterized in terms of morphological and microscopic characteristics (Figure 7). Morphological examination showed that the mycelia mat was purely white, the odor was absent and growth was initiating

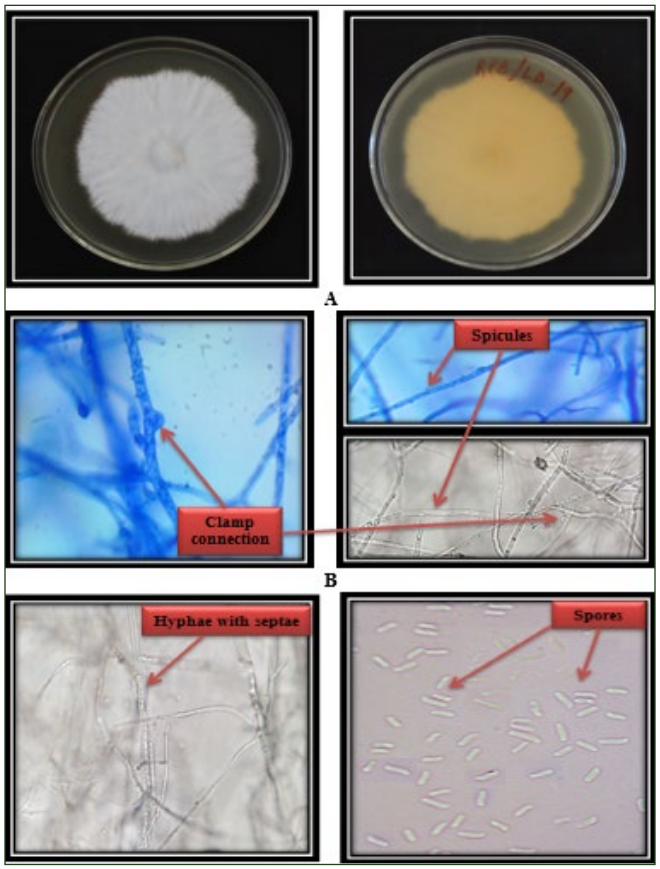

Figure 7: Morphological characters of LD19 fungi; A. Morphology; B. Microscopy (Clamp connection and Spicules); C. Microscopy (Hyphae and Spores)

near the inoculum and spreading throughout the surface of the medium.

Microscopic examination showed that the presence of hyaline and septate hyphae with clamp connections, ellipsoidal spores and thin, short spicules. The ITS primer amplified gene sequence of the LD19 isolate was searched for sequence homology using BLAST tool on NCBI database and revealed that strain LD19 showed 100% similarity with *Schizophyllum commune.* The sequence was submitted to NCBI GenBank with accession No. MW720154. Based on phylogenetic analysis and microscopic examination, lignin-degrading fungi LD19 was identified as *Schizophyllum commune* (Figure 8).

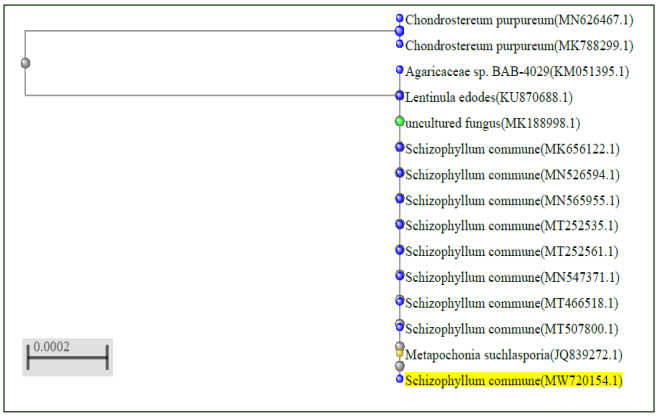

Figure 8: Phylogenetic tree of LD19 (*Schizophyllum commune*)

Likewise, Tullioet al., (2008) reported the presence of hyaline and septate hyphae in *Schizophyllum commune* with clamp connections and short, thin spicules. Kumar et al., (2018) identified *Schizophyllum commune* (NI-07) using ITS/5.8S rRNA gene sequence with cylindrical to ellipsoidal smooth white spores, hyphae having septae and clamp connections. Vijya and Reddy (2012) also identified mushroom fungi as *Schizophyllum commune* with cellulolytic and ligninolytic activity. Singh et al., (2017) identified two ligninolytic fungi DMRF-7 and DMRF-8 as *Schizophyllum commune* and *Pezizomycotina* sp., respectively through blasting of the sequences of Internal Transcribed Spacer (ITS) region of 5.8S rRNA gene from fungal isolates. Aswalet al., (2020) also identified lignin-degrading white-rot fungal isolate PSS2 as *Schizophyllum commune.*

4. Conclusion

Based on the results of the current study, among the 8 selected fungal strains, 5 produced all the three enzymes *viz.* laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP), 2 produced two enzymes while 1 produced only one enzyme. LD19 showed higher potency index as well as lignin modifying enzyme activity and identified as *Schizophyllum commune* by morphological, microscopic and molecular characterization.

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