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RESEARCH ARTICLE

SCREENING OF FUNGI ISOLATED FROM DIFFERENT REGIONS OF KERALA FOR LIGNIN PEROXIDASE ACTIVITY

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ABSTRACT

Fungi produce lignolytic enzymes for utilizing the wood components; the important one among them is lignin peroxidase (LiP). The present study focuses to find out the highest lignin peroxidase producer among the collected fungi, isolated from in and nearby areas of Western Ghats and other parts of Kerala. Among the 62 isolates, the highest producer was identified to be *Endomelanconiopsis endophytica* LP01 from the regions of Western Ghats and this is the first report that demonstrates production of LiP in *Endomelanconiopsis* spp. The isolates from the Western Ghats has got highest LiP activity compared to those from other regions. The qualitative assay for LiP employing azure B supplemented in Potato Dextrose Agar as substrate showed that the LiP Produced by *E. endophytica* LP01 could oxidize the dye, which would extend its role in textile and other industries as well.

Key words: Western Ghats, Lignin peroxidase, Lignin, *Endomelanconiopsis endophytica*, and Azure B.

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INTRODUCTION

Fungi actively produce several oxidative and hydrolytic enzymes for degrading wood and other lignocellulosic biomass (Liers*et al*., 2011). The Western Ghats is a mega biodiversity region and found to be inhabited with diverse fungi (Selvam*et al*., 2012; Menakshisundram M and Bharathiraja C, 2013) having the potential of wood degradation. Wood is composed of cellulose, hemicellulose, lignin and extractives. The presence of lignin makes the plant biomass highly recalcitrant due to the complex aromatic ring structure. Different groups of fungi such as white rot fungi (Eriksson *et al*., 1990; Leontievsky*et al*., 1994; Arora and Gill, 2004), Brown Rot Fungi (Kirk and Adler, 1970; Machuca and Ferraz, 2001), and soft rot fungi (Rodriguez *et al*., 1996) possess the enzymes to achieve degradation. Endophytic fungus has also been reported to have lignin degrading enzymes (Urairuj*et al*., 2003; Choi *et al*., 2005). Lignin peroxidase (LiP) is an extracellular enzyme produced by fungus to utilize the biomass for its growth and survival. LiP is a heme peroxidase whose active site is homologous to the other peroxidases (Ropp *et al*., 1991). It drives the oxidation of phenolic and non phenolic structures (Kirk and Farell, 1987). It catalyzes hydroxylation of benzylic methylene groups, phenol oxidation, oxidation of benzyl alcohols to aldehydes or ketones, aromatic ring cleavage of non-phenolic lignin model compounds and Cα-Cβ cleavage of the propyl side chains of lignin, (Renganathan *et al*., 1985; Umezawa and Higuchi 1987; Chung and Aust 1995). The LiP family include multiple isozymes with a molecular weight ranging from 38 kDa to 43 kDa, which differ in their isoelectric points (Glumoff *et al*., 1990).The native enzyme is oxidized by H_2O_2 which generates two-electron deficient compound I followed by one electron deficient Compound II and subsequent oxidation restores the peroxidase to its native resting stage. The excess H_2O_2 will combine with compound II of LiP and generates an inactive form compound III (Niladevi 2009). LiP can be considered as a promising enzyme for biotechnological applications since it has got wide applications in different areas including food industry (Lessen Messen *et al*. (1996); Barbosa *et al*. (2008)), paper and pulp industry (Sigoillot *et al*., 2005), Textile industry (Rodriguez *et al*., 2005), and degradation of xenobiotics and pesticides (Abraham *et al*., 2002; Gomes *et al*., 2009). The present study aims to isolate and identify the fungus with highest Lignin peroxidase activity.

MATERIALS AND METHODS

Sources of organisms

Sixty two fungal strains were isolated from tree barks, litter, and soil of Western Ghats and other locations of Kerala during rainy season. The strains were cultured on Potato dextrose agar

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(PDA) plates at room temperature, till it attains full growth. Slants of these strains were also made and stored at 4˚C, which were sub-cultured at regular intervals.

Substrate preparation: Dried paddy straw without any mold symptoms was selected as the substrate for solid state fermentation. The straw was chopped into small pieces and weighed 4g into conical flasks. To the prepared substrate, 10 mL citrate buffer (0.1M) was added for adjusting the moisture content. The substrate was sterilized by autoclaving for 20 min at 121°C and 15 lb/inch^2.

Inoculum preparation and enzyme production: Four agar plugs of size 0.5 cm^2 was used as inoculum to the prepared substrate and incubated at room temperature for 7 days.

Enzyme extraction: The extracellular enzyme was extracted by adding 50 mL of 0.1 M citrate buffer (pH 5) to the seven day incubated culture and the crude enzyme was harvested by centrifugation (Sigma 3K3O) at 8000 rpm for 10 minutes at 4˚C.

Lignin peroxidase assay: Lignin Peroxidase activity was determined by H_2O_2 dependent veratraldehyde formation from veratryl alcohol at 310 nm (Tien and Kirk, 1984) using spectrophotometer (Shimadzu UV 1800). The reaction mixture contains 0.25 mL of enzyme solution, 0.25 mL 1mM veratryl alcohol, 0.2 mM H_2O_2 and 0.5 mL of 0.1M citrate buffer. The enzyme activity was expressed in IU/mL. An International Unit (IU) is defined as the amount of enzyme activity which will catalyze the transformation of 1 micromole of the substrate per minute under standard conditions.

Statistical analysis: Experiments were performed in triplicate and the LiP activities were analyzed statistically by ANOVA using SPSS software version 16.0 (SPSS Inc, USA). Relative activities of different fungi were analyzed using Least Significant Difference for test of significance. Differences in means were considered significant at $P<0.05$.

Molecular characterization and identification: Of the sixty two isolates, the one with highest lignin peroxidase activity was selected and the genomic DNA was isolated according to modified Gupta *et al*., (2013).

The DNA of the isolate with highest lignin peroxidase activity (PKD 24) was amplified with ITS 1(forward primer: 5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4 (reverse primer: 5'-TCC TCC GCT TAT TGA TAT GC-3') primers and sequenced. The reagents used for PCR (Polymerase Chain Reaction) were 1µL of DNA template, 2µL of 10X PCR buffer, 1.5 mM $MgCl_2$, 200 µM each dNTP, 0.5µM primers, 1 unit Taq polymerase (Sigma-Aldrich, USA) and double distilled water. The PCR was run using Bio- Rad MJ mini thermocycler. The PCR conditions were as follows: denaturation for 2 min at 95˚C, followed by 35 cycles of denaturation for 30 sec at 95˚C, annealing for 2 min at 57˚C and extension for 1 min at 72˚C. The final extension was done at 72˚C for 10 min. The PCR amplified product was run on 2% agarose gel stained with ethidium bromide (EtBr) and visualized under UV light. The photograph of the gel was taken using gel documentation system (Syngene). The sequencing of the PCR product was carried out using ABI 3730XL DNA sequence.

Phylogenetic analysis: The nucleotide BLAST program of NCBI was used to identify the organism based on homology. The sequence in FASTA format was submitted for the analysis and the sequences were aligned based on heuristic approach. The sequence with highest percentage of identity and sequence query coverage was selected and identified as the organism under study. The sequence of the other hits showing 99% identity and 100% query coverage were selected for tree construction. The selected hits (listed in table 1) with the identified sequence were phylogenetically analyzed using the software MEGA 6.06 by neighbor joining method. Bootstrapping was done with 500 replications.

Growth analysis: To study the growth pattern of the identified strain, a 0.5 cm^2 plug was cut from the edge of the colony and placed on the PDA medium. The inoculated plate was kept for incubation at room temperature. The growth was monitored on each day.

Microscopy: The identified fungus with highest LiP activity was stained with lactophenol cotton blue and observed under microscope.

Qualitative assay: The organism was grown on Potato Dextrose Agar medium supplemented with 0.0025% azure B (Ali *et al*., 2012). The color change was noted.

Table 1. List of Selected hits for Phylogenetic analysis

Sl No.	Organisms	Accession No.
1	Fungal endophyte culture-collection STRI:ICBG-Panama:TK1396	KF435917.1
2	Fungal endophyte culture-collection STRI:ICBG-Panama:TK1152	KF435491.1
3	Fungal endophyte culture-collection STRI:ICBG-Panama:TK119	KF435346.1
4	Fungal endophyte culture-collection STRI:ICBG-Panama:TK1393	KF435222.1
5	*Endomelanconiopsis*sp. NF79	KJ588255.1
6	*Endomelanconiopsis* sp. F4801	KF746076.1
7	*Endomelanconiopsisendophytica*strain CMW28552	GQ469968.1
8	*Endomelanconiopsisendophytica*strain CMW28618	GQ469966.1
9	*Endomelanconiopsisendophytica*strain CMW28563	GQ469965.1
10	Fungal endophyte isolate 1980	EU687005.1
11	Fungal endophyte isolate 1973	EU687003.1
12	Fungal endophyte isolate 1201	EU686820.1
13	Fungal endophyte isolate 1173	EU686814.1
14	Fungal endophyte isolate 1119	EU686806.1
15	Fungal endophyte isolate 1117	EU686800.1
16	Fungal endophyte culture-collection STRI:ICBG-Panama:TK1324	KF435357.1
17	*Endomelanconiopsisendophytica*strain CBS 120397	KF766164.1
18	*Endomelanconiopsisendophytica*strain Q1414	FJ799942.1
19	Fungal endophyte culture-collection STRI:ICBG-Panama:TK635	KF436304.1
20	*Endomelanconiopsisendophytica* strain CMW28551	GQ469967.1
21	*Endomelanconiopsisendophytica*strain CBS 122543	EU683670.1
22	Fungal endophyte strain LA73	JX156072.1

Sixty two strains of fungi were isolated and maintained on PDA. Among the screened fungi, those showing highest positive LiP activity were found to be isolated from the regions in and around Western Ghats. The highest activity was shown in PKD 24 (17.57±3.65 IU/mL), followed by PKD 25 (15.57±6.52 IU/mL) and PKD 16 (14.63±3.90 IU/mL). The least activity was found to be in TVM 3B (0.17±0.15 IU/mL) and others possess positive LiP activity neither strong nor weak. Among them the one with highest activity was selected for genomic DNA isolation.

Table 2. The LiP activities of the isolated fungi

Sl No.	Organism	Location	LiP (IU/mL)
1	DF 12	Ernakulam	4.87±5.14
2	PKD 14	Palghat	0.77±0.89
3	TVM 5A	Trivandrum	0.90±0.52
4	PKD 7	Palghat	1.17±0.61
5	TVM 3	Trivandrum	1.00±0.40
6	CST 2	Ernakulam	1.33±1.40
7	TVM 4A	Trivandrum	0.30±0.20
8	CST 4	Ernakulam	4.57±4.72
9	DF 8	Ernakulam	1.20±1.21
10	TVM 7B	Trivandrum	4.17±3.35
11	AN	Palghat	2.57±2.63
12	DF 9	Ernakulam	0.50±0.40
13	Neli 3	Palghat	1.47±1.61
14	PKD 13	Palghat	4.97±7.90
15	DF 7	Ernakulam	0.90±0.20
16	DF 2	Ernakulam	0.77±0.51
17	PKD 1	Palghat	1.30±0.65
18	DF 3	Ernakulam	2.93±2.40
19	CST 5	Ernakulam	1.90±1.73
20	TVM 6	Trivandrum	3.90±3.20
21	DF 6	Ernakulam	2.03±0.80
22	PKD 21	Palghat	4.47±5.96
23	Neli 4	Palghat	1.20±1.15
24	DF 11	Ernakulam	2.27±2.00
25	PKD 11	Palghat	1.17±0.63
26	TVM 3C	Trivandrum	0.73±0.61
27	PKD 9	Palghat	0.90±0.60
28	Vakka	Ernakulam	1.87±2.04
29	TVM 1A	Trivandrum	0.57±0.64
30	PKD 12	Palghat	0.93±1.13
31	TVM 8A	Trivandrum	4.53±1.15
32	TVM 3B	Trivandrum	0.17±0.15
33	Neli 2	Palghat	3.70±2.47
34	PKD 20	Palghat	2.73±1.30
35	TVM 7c	Trivandrum	1.40±0.65
36	PKD 19	Palghat	4.37±0.55
37	PKD 2	Palghat	1.03±0.90
38	PKD 5	Palghat	0.40±0.30
39	Neli 4	Palghat	1.30±1.21
40	PKD 15	Palghat	1.30±0.20
41	TVM B	Trivandrum	0.80±0.20
42	TVM 2A	Trivandrum	2.63±1.07
43	TVM 9	Trivandrum	0.87±0.86
44	PKD 6	Palghat	0.93±0.75
45	PKD 18	Palghat	1.70±1.41
46	TVM 6c	Trivandrum	1.30±0.65
47	TVM 2B	Trivandrum	1.37±0.77
48	PKD 17	Palghat	0.83±0.80
49	PKD 8	Palghat	1.63±0.89
50	DF 4	Ernakulam	6.97±6.07
51	PKD 16	Palghat	14.63±3.90
52	CST 3	Ernakulam	9.80±8.54
53	DF 1	Ernakulam	10.83±9.70
54	PKD 3	Palghat	8.17±5.45
55	PKD 10	Palghat	12.47±4.50
56	PKD 4	Palghat	7.27±3.26
57	PKD 22	Palghat	0.57±0.55
58	PKD 23	Palghat	1.23±1.02
59	PKD 26	Palghat	0.33±0.40
60	PKD 27	Palghat	1.27±0.92
61	PKD 24	Palghat	17.57±3.65
62	PKD 25	Palghat	15.57±6.52

PCR amplification was carried out using primer pair ITS1-ITS4 and the product of 600bp was obtained. The corresponding band was eluted, sequenced and submitted to Genbank (Genbank Accession Number: KP125488). According to the BLAST output, the molecular identified organism was indicated to be *Endomelanconiopsis endophytica* LP01. This is the first report that demonstrates *Endomelanconiopsis spp* produce LiP for oxidizing lignin to utilize their substrate for growth. The particular strain isolated belongs to Botryosphaeriaceae and researches have reported that some Botryosphaeriaceae members produce LiP (Ali *et al*., 2014).

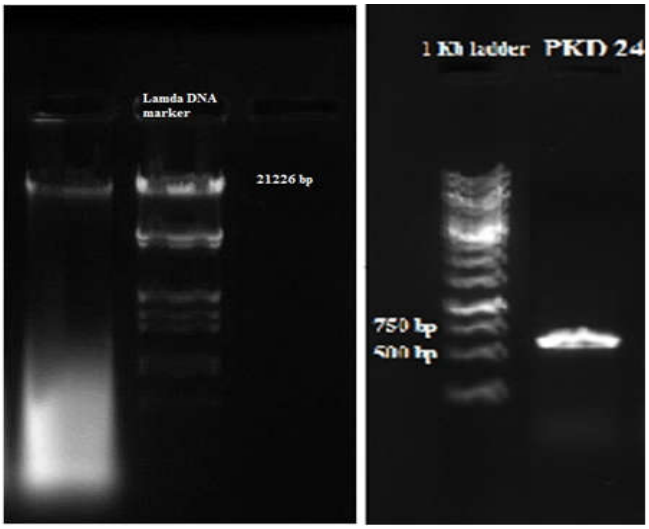

Fig 1. The isolated DNA on left side (lane 1: PKD 24 DNA, lane 2: Lamda DNA EcoRI/ Hind III double digest) and PCR product of 18 s ribotyping on right side (lane 1: 1kb ladder and lane 2: 600bp product)

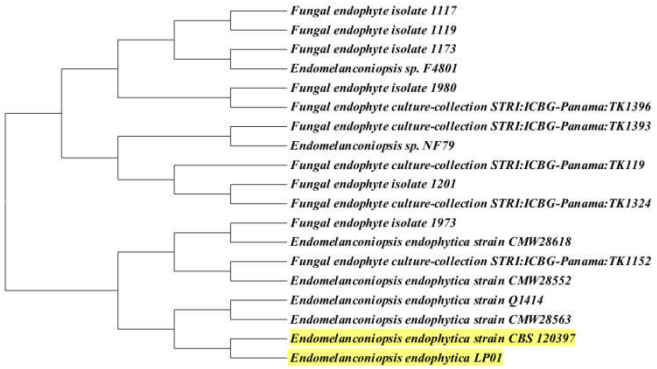

Fig. 2. Phylogenetic tree showing the relationship between the *E. endophytica* LP01 with other hits having 99% identity

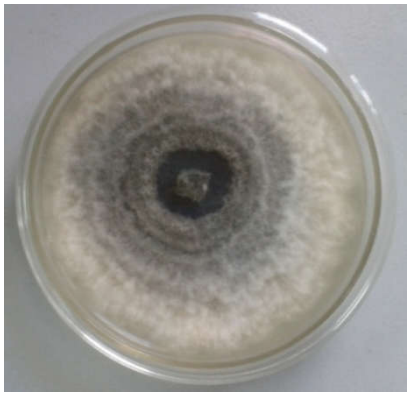

Fig 3. Fifth day culture of *Endomelanconiopsis endophytica* LP01on PDA

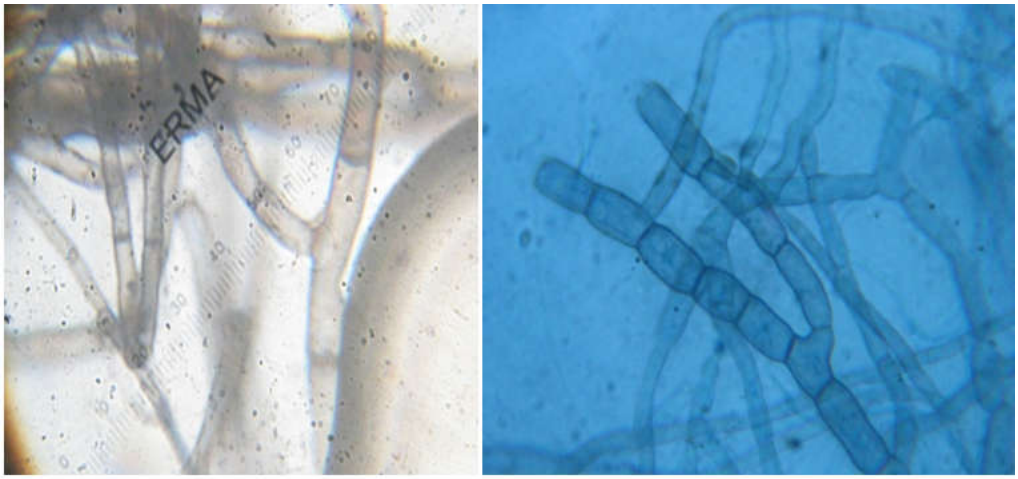

Fig 4. Septate hyphae of *Endomelanconiopsis endophytica* LP 01 at 100X

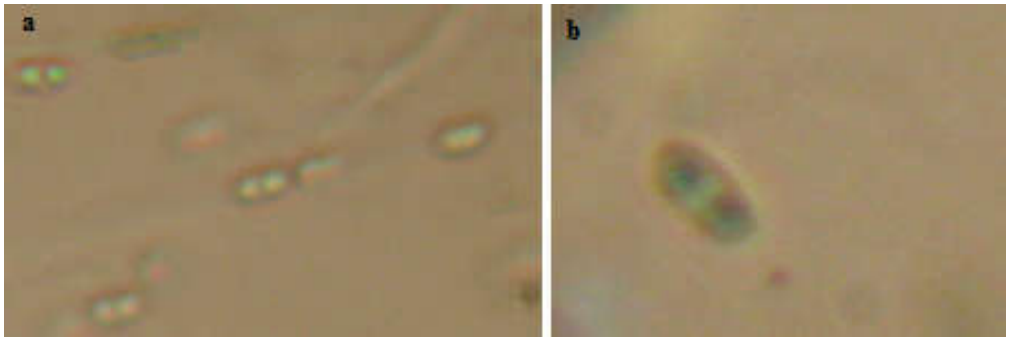

Fig 5. a) microconidia and b)macroconidiaof*Endomelanconiopsis endophytica* LP 01 at 40X

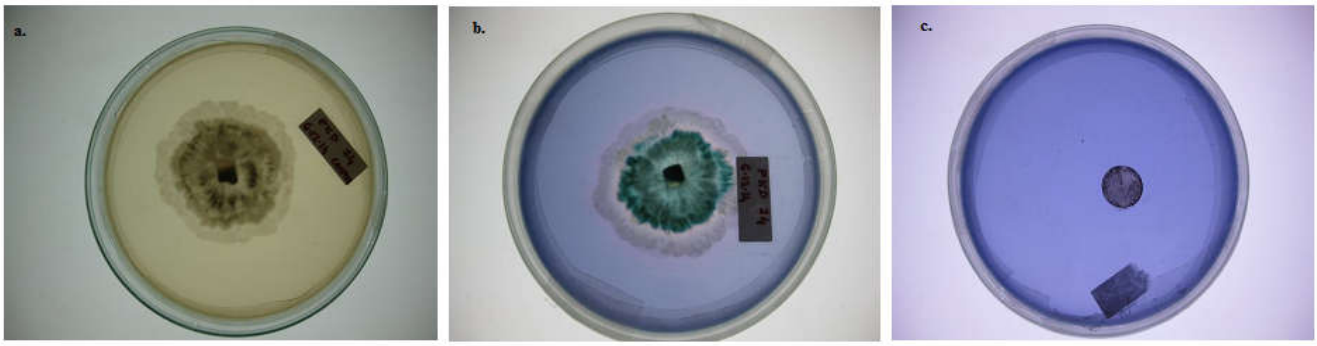

Fig 6. Oxidation of azure B by *Endomelanconiopsis endophytica* LP 01 on 3rd day; a. *Endomelanconiopsis endophytica* LP 01 on PDA, b. *Endomelanconiopsis endophytica* LP 01on PDA supplemented with azure B, c. Control plate without fungus

From the BLAST output, the sequence of other hits with 99% identity and 100% query coverage were selected. The selected hits with *E. endophytica* LP01were phylogenetically analyzed using the software MEGA6. *E.endophytica* LP01 shares homology with *Endomelanconiopsis endophytica*strain CBS 120397 (Fig 2). Both of them belongs to same clade suggesting that the *E. endophytica* LP01 has got point mutations from the other *Endomelanconiopsis* spp. *Endomelanconiopsis endophytica* LP01was stained with lactophenol cottonblue and observed under microscope. The stage micrometer and ocular micrometer was calibrated. The strain was found to have septate hyphae with a width of 6.3µm (Fig 4). *Endomelanconiopsis endophytica* LP01 was cultured on PDA and the growth was observed. Colonies on PDA appears to be dark gray to black in color (Fig 3). At first the colonies were found to be colorless but after the third day, the colonies were found to grow in concentric irregular manner with centre portion gray in color while the periphery colorless. Qualitative assay is a powerful technique to inspect the production of LiP enzyme by the organism.

The PDA medium is supplemented with 0.0025% azure B, which offers the medium purple color. On the third day, the purple color gets converted to green color by the oxidation of azure B by LiP, which indicates the start of LiP production in *Endomelanconiopsis endophytica* LP01 (Fig 5). The peripheral areas does not show green color, which establishes the fact that LiP is a secondary metabolite.

DISCUSSION

Delignification by oxidative enzymes produced by fungi helps to degrade wood and for utilizing the biomass, which is hindered by the presence of recalcitrant aromatic polymer lignin. Most of the basidiomycetes fungi have shown to produce lignin peroxidase and a very few endophytes utilize lignin. The studies reported that most of the endophytes may not have the ability to degrade lignin (Carroll and Petrini, 1983) but the present study investigated the production of lignin peroxidase by an endophyte, which could degrade lignin. Among the 62 isolates collected from different regions,

the isolates from Western Ghats showed the highest LiP activity compared to other regions. Forest ecosystems are inhabited with many wood rotting fungi. As the Western Ghats has got a great floral biodiversity, which might get added to the litter and it has been reported that litter harbours diverse fungi (Geethanjali, 2012). The litter has sufficient quantity of lignin and other biopolymers contributed by the plant residues (Rao, 2008). As the floral diversity increases the litter content in the soil would also increase, which would offer more lignin richness in the same region. This could be the reason for the highest LiP activity in the isolates from Western Ghats. Fungal identification can be achieved by ITS primers, which exploits the conserved regions of 18S, 5.8S and 28S rRNA genes for amplifying the noncoding regions present between them (White *et al*., 1990). In the present study, ITS1 and ITS 4 were the primers used for identification purpose and the isolated strain was found to be *Endomelanconiopsis endophytica* LP01, which is an endophyte belonging to botryosphaeriaceae family under Ascomycetes.

Ascomycetes fungi have shown to degrade lignin and some of them had reported to produce LiP also (Gao *et al*., 2011). Some of the Botryosphaeriaceae members have shown to produce LiP (Ali *et al*., 2014) and other lignin modifying enzymes (Castilho *et al*., 2009). The identified strain *Endomelanconiopsis endophytica* LP01 cultured on PDA appears to be dark gray to black in color and the colonies grow in concentric irregular manner with centre portion gray while the periphery colorless. This was in accordance with the same pattern of growth observed by Rojas *et al*., 2008. The organism was found to be a slow growing fungi. Azure B is a non phenolic dye and a relatively refractory synthetic compound, which cannot be decolorized physically or biologically under usual conditions (Archibald, 1992). It has been designed for granting stability to textiles and its a problem waste for the industry (Anliker, 1979). In the present study, Azure B was used for the qualitative analysis of the strain and significant oxidation of the dye was observed. As the strain compass the potential to oxidize the dye, which could be used for treating the textile effluent with azure dyes. Fungal endophytes have been shown to produce novel compounds, which have economical value in various fields (Naik*et al*., 2008). The LiP production by the particular strain was not studied and reported so far. Thus the LiP form *Endomelanconiopsis endophytica* LP01 would offer exciting applications in various industries such as textiles, paper and pulp industry, dye decolorization and even in bioremediation.

Conclusion

The best LiP producer was screened from 62 fungal isolates from Western Ghats and other parts of Kerala. The organism was identified to be *Endomelanconiopsis endophytica* LP01 and the enzyme activity was 17.57±3.65 IU/mL. The LiP enzyme produced by the particular strain could oxidize azure B which is a problematic waste in textile industry.

Conflicts of interest

The authors declare that there is no conflict of interest

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