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Screening and Molecular Identification of Potential Lignolytic White Rot Fungi Isolated from Western Ghats

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The mounting concerns over the usage of enormous amount of wood and perilous chemicals in paper industry have led the researchers to develop ecofriendly green technologies for pulp and paper production. Lignocellulosic agro-waste can be an excellent alternative to the wood in papermaking. White rot fungi with ligninolytic properties can be effectively used for pretreatment of agro-wastes for pulp making. This study isolates and identifies previously unexplored strains of white rot fungi from Western Ghats of Kerala, India which can be used for developing biological systems for lignocellulosic treatment in pulp and paper production. Ten isolates were identified using molecular techniques and screened for their extracellular enzyme activities, including laccase, Manganese peroxidase and Lignin peroxidase. Among the 10 isolates, Trametes versicolor (Laccase activity 31.79 U/ml, Manganese peroxidase (MnP) 42.336 U/ml and Lignin peroxidase (LiP) - 50.65U/ml, Favolus teniculus (Laccase - 41.54 U/ml, MnP - 44.07 U/ml &LiP - 30.54), Coriolopsis byrsina (Laccase - 42.56 U/ml, MnP - 43.54 U/ml and LiP - 25.14 U/ml) Lenzitus betulina (Laccase - 37.15 U/ml, MnP - 38.97 U/ml and LiP - 30.43 U/ml) exhibited highly promising lignolytic enzymatic system. The study's findings may provide a better eco-friendly substitute for conventional chemical treatments in various industrial applications.

Keywords: Coriolospsis byrsina; Favolus teniculus; Lignin; laccase; Lignin peroxidase; Lenzitus betulina; Manganese peroxidase.

Lignocellulosic waste generated from forestry and agricultural practices constitutes a significant portion of municipal solid wastes and causes substantial environmental pollution. The escalating demands for wood resources in the paper making industry and the use of hazardous chemicals in paper production pose additional threat to the environment. Fungi, especially white rot fungi are recognized as the major organisms for lignocellulosic degradation [1,28]. White rot fungi are the major organisms which are capable of effective lignin mineralization. Efficient removal of lignin from lignocellulosic wastes makes them a better alternative for wood in paper making.

Pretreatment of lignocellulosic wastes using microorganisms and their enzymatic machinery can efficiently break down lignin and alter lignocellulose. White rot fungi is regarded as the greatest promising microorganism which can mineralize lignin to CO_2 and water in pure culture[3]. White rot fungi produces variety of extracellular lignolytic enzymes such as Laccase(EC1.10.3.2)

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and peroxide reductases or peroxidases which includes Manganese peroxidase- MnP (EC 1.11.1.13), Lignin peroxidase- LiP (EC1.11.1.14) and versatile peroxidase (EC1.11.1.16) [1,4]). Lignin modifying enzymes are the product of secondary metabolism of white rot fungi.

In recent years, the studies on lignin modifying white rot fungi have been increased in search of efficient lignolytic machinery to utilize lignocellulosic biomass more effectively. The lignolytic properties of white rot fungi can be employed in many aspects of lignolytic biomass pretreatment such as Bioethanol production[9], pulp and paper making [11,12] and bioremediation of xenobiotic compounds[13]. The capability of white rot fungi to generate lignolytic enzymes is the key focus of these studies. Various white rot fungi have been reported to exhibit lignin degrading ability. Previous studies have mainly focused on *Phenerochete chryosporium*, *Cereporiopsis subvermispora*, *Trametes versicolor, pleorotus ostreatus*[5,6,7,8] as the widely studied white rot fungi.

However, the identification of alternative and unexplored strains with potential lignolytic abilities is critical for the development of feasible biotechnological systems for various industrial applications and proposing a more eco-friendly substitute for conventional chemical treatments. Western Ghats are the hotspots of biodiversity with varied flora and fauna. In this study fruit bodies have been collected from Western Ghats of Kerala and it is expected to provide unexplored candidates with potential lignolytic machinery for various biotechnological applications.

Thus, the present study aims to (i) isolate white rot fungi from Western Ghats, molecular identification of the strain ii) screening of this isolates for its ability to degrade lignin in order to find unexplored strains with high lignin degrading system.

MATERIALS AND METHODS

Collection and culturing of fungal samples

The samples including fruiting bodies from lignocellulosic rich litter piles and decayed woods from Vellanikkara area of (10.5449° N, 76.2864° E) Western Ghats were collected in sterile zip lock polythene bags and brought to the laboratory for further analysis.

Total number of 10 fruiting bodies were collected. The collected samples were cultured in MGYP broth (3 g Malt extract, 10g Glucose, 03g Yeast extract, 05 g Peptone per litre of distilled water) and Malt Extract Agar medium. The fruiting bodies were cut into pieces and surface sterilized with 70% ethanol inoculated in 25 ml of MGYP medium. It was then kept under room temperature for 4 to 6 days[14]. The pure culture was then maintained by subculturing in 2%Malt Extract Agar medium.

Molecular identification of fungal samples

DNA isolation was done by CTAB method.[14.] The Isolated DNA was then amplified by using I8S rRNA primers ITS1 (52 -TCCGTAGGTGAACCTGCGG-32); ITS4: (52 -TCCTCCGCTTATTGATATGC-32). The reaction mixture consisted isolated DNA-2µl, ITS primers-1µl each, pcr master mix - 10µl, and sterile water -8µl. PCR cycles have been carried out with an initial denaturation temperature of 95^0C for 2 minutes and the other conditions were: denaturation - 95^0C - 1 minute, annealing - 55^0 C -1 minute, extension - 72^0C - 1 minute and final extension at 72^0C for 5 minutes. The total number of cycle was 30. The amplicon obtained was electrophoresed using 1.2% agarose gel and imaged in a gel documented. The product was then purified using purification column and sequenced (Chromus Biotech, Banglore). The sequence obtained was deposited to NCBI databank to get the accession number and it was analyzed using BLAST, a NCBI tool. The sequences were compared with the similar sequences available in the Genbank by Multiple sequence alignment using the tool ClustalW. The aligned data was used to construct phylogenetic tree using the software MEGA7.

Primary screening

Dye decolourization method using Methylene Blue

The fungal culture was inoculated in malt extract agar plates containing 0.01% Methyelene blue as described by Sasikumar et al. with slight modification[24] and incubated at 25ºC for two weeks. A clear zone formation around the colony will indicate the presence of enzyme Lignin peroxidases.

Guaiacol agar plate assay

Malt extract agar plates with 0.01% guaiacol were prepared and the fungal culture was

inoculated. It was then incubated at 25ºc for two weeks. Positive reaction will form brown color which indicates the presence of Laccase enzyme[15].

Secondary screening by quantitative Enzyme assays

Fungal mycelium discs of 6mm were cultured in 20 ml of Mineral Salt medium and incubated for 12 days (composition g/L: Glucose-10g/L;Pottasium dihydrogen orthophosphate -2 g/L;Magnesium sulphate hepta hydrate -0.5 g/L;Calcium chloride-0.1 g/L; Ammonium tartrate -0.2 g/L. Trace element solutioncomprised of (mg/L) Ferrous sulfate-12;Manganese sulphate-3;Zinc sulfate- 3;Cobalt sulfate hepta hydrate -1;Ammonium molybdate -1(pH. 5.0)[16]. After incubation the culture was filtered using Whatman No1 filter paper. Around 5 ml of supernatant was used to analyze the extracellular enzymes. All the experiments were done in triplicates.

Laccase (E.C.1.10.3.2)

For determining the activity of laccase enzyme, guaiacol(2 mM) was used as the substrate. Reaction mixture was prepared using Guaiacol(2 mM) in sodium acetate buffer (pH 4.5). 100 µl of enzyme was added and the mixture was incubated at 30ºC. Absorbance was taken at 450 nm. The quantity of laccase enzyme needed to oxidize 1µm of guaiacol per minute[15] is regarded as one unit enzyme activity. The extinction coefficient of guaiacol at 450 nm is 12,100 $M^{-1}cm^{-1}$

Lignin peroxidase(EC.1.11.1.14)

LiP enzyme activity was analyzed according to Tien & kirk method (1989)[25]which relies on the rate of oxidation of veratryl alcohol. The reaction mixture constitutes 250 µl of sodium tartarate buffer, 100 µl of veratryl alcohol(2 mM) and 100 µl of supernatant. The reaction was commenced by the adding of 100 µl H_2O_2. The absorbance was taken at 310 nm. One unit of enzyme activity is expressed as the quantity of enzyme required to oxidize 1µm of veratryl alcohol/minute.

Manganese peroxidase (E.C.1.11.1.13)

Manganese peroxidase assay was carried out based on the oxidation of guaiacol. The reaction mixture consists of 100 µl of sodium tartarate buffer (pH 5.0), 10 µl of guaiacol, 10 µl of $MnSO_4$ and 100 µl of enzyme. Addition of 10 µl of H_2O_2 commenced the reaction and absorbance was measured at 465 nm[15].

RESULT AND DISCUSSION

White rot fungi which produces ligninolytic enzymes are important biological tools with possible application in wide variety of fields. The search of strong lignin degrading enzymatic machinery has always been an interesting topic among researchers owing to its potential

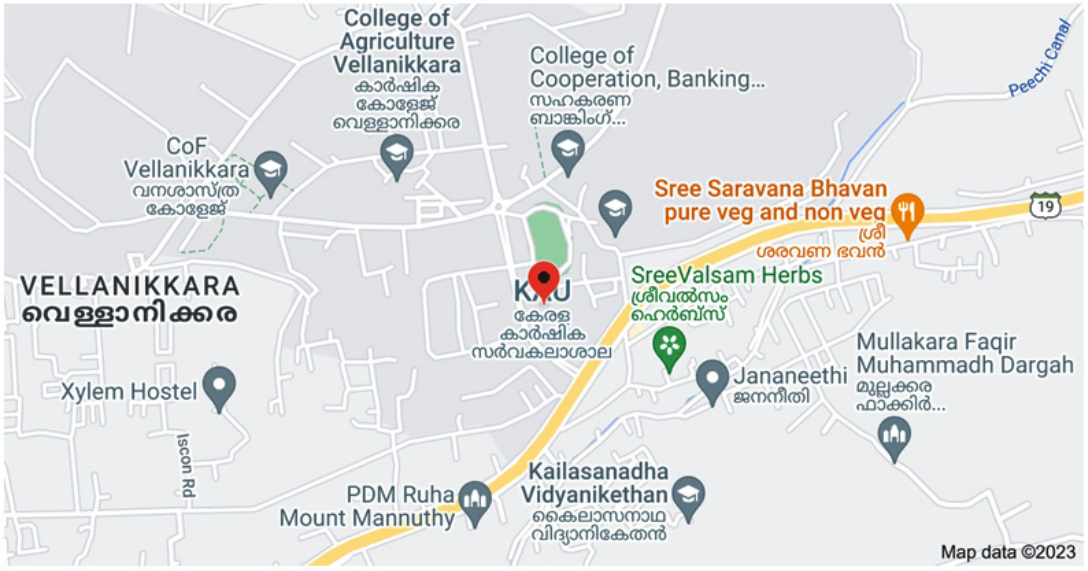


Fig.1. Location map of site of sample collection

applications. In this study 10 samples from Western Ghats have been isolated and identified.

The biodegradation ability of saprophytic basidiomycetes is closely connected with the production of extracellular enzymes, among them, Laccase, Lip, MnP plays important roles[17]. Some wood degrading fungi produces all these three enzymes, while others produces only one or two of these enzymes.[6]. These enzymes are not only involved in lignin degradation as observed in the delignification of natural lignocellulosic materials, but also actively degrade many recalcitrant compounds including dyes[18].

Primary screening for strains producing lignolytic enzymes

The initial screening strategy used for detecting the capability of fungal strains to produce lignin degrading enzymes was the dye decolourizing technque[18]. The fungi capable of producing lignolytic enzymes which decolorizes the azo dye Methylene blue were screened. During dye degradation, the azo linkage is cleaved by oxidation by lignolytic enzymes like laccase, MnP, LiP produced by the white rot fungi[19]. Lignolytic enzymes are oxidative in nature and hence are able to decolorize basic dyes by reduction.

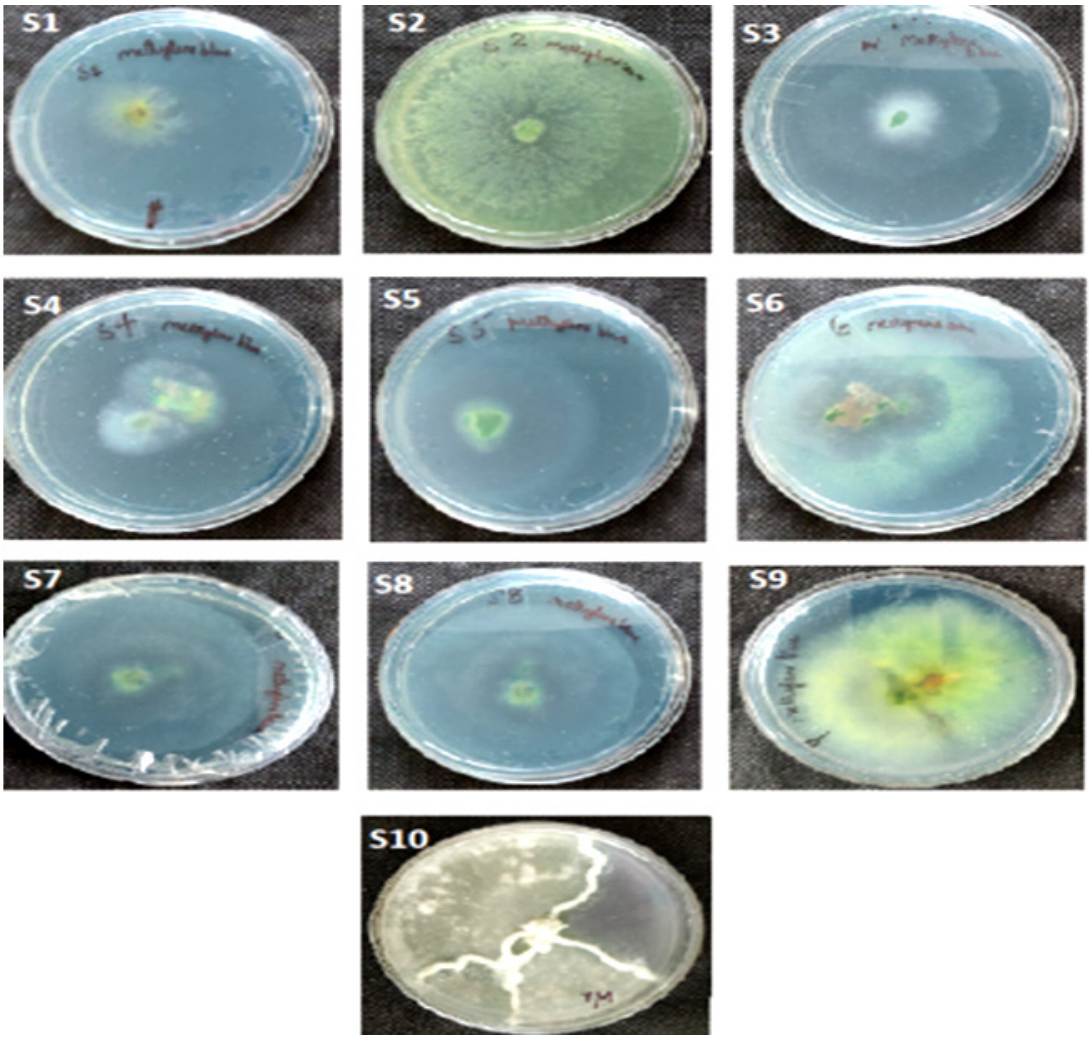


Fig. 2. Plates showing degradation of methylene blue. Clear zones around the colonies indicate the positive reaction.

In the study, all the isolates were positively responded to the dye decolorization assay using methylene blue. Clear zones were formed around the colonies indicating the presence of lignin modifying enzymes. (Figure 2). This suggests all the isolates are able to produce any of the oxidative lignolytic enzymes. In line with the studies by Sasikumar et al. [24], the decolorization of methylene blue to clear zones can be used as indicative of production of Lignin peroxidase enzyme.[24.] This suggests all the ten isolates are capable of producing Lignin peroxidase enzyme.

The ability of fungi to produce lignolytic enzymes can be verified by its ability to oxidize phenolic compounds like guaiacol and syringaldazine[21]. 10 samples were tested for the ability to oxidize guaiacol, to screen potential isolates which degrade lignin.

All the isolates formed reddish brown color zone around the colony, indicating the

Table 1. Primary screening of isolates for detecting the presence of lignolytic enzymes

Isolate	Name of the strain	Primary screening	
		Methylene blue test - zone of clearance (mm)	Guaiacol plate test-reddish brown zone(mm)
CBNR 1	*Trametes versicolor* (OL636386)	3	4
CBNR 2	*Coriolopsis byrsina* (OL587502)	5	2
CBNR3	*Lenzitus betulinus* (OM424452*)*	7	4
CBNR 4	*PhenerochaeteSp* (OL636387)	6	2
CBNR 5	*Phenerochaete avellanea* (ON945561)	5	3
CBNR 6	*Ganoderma austral* (OL636391)	10	8
CBNR 7	*Rickenella fibula* (OL636388)	12	6
CBNR 8	*Favolus teniculus* (OL636390)	12	2
CBNR 9	*Coriolopsis byrsina* (ON945562)	12	2
CBNR 10	*Coriolopsis byrsina* (OL539546)	12	6

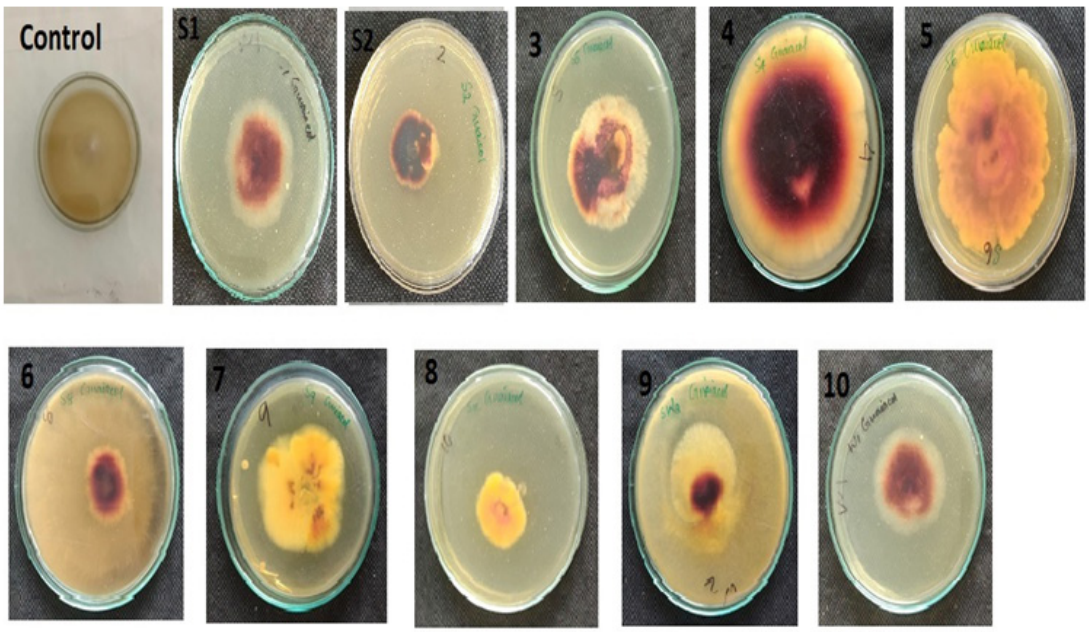


Fig. 3. Plates showing reddish brown zones indicating the oxidation of guaiacol

presence of laccase enzyme. (figure2). Production of reddish brown zone is due to the oxidation of guaiacol by laccase enzyme[15]. The similar results reported by Anuja&Aggarwal et al.[15] and Dhoib et al.[18]in well studied white rot fungi such as *Phenerochaete chryosporium, Trametes versicolor*, *Pleurotus ostreatus*, *Ganoderma lucidum.*

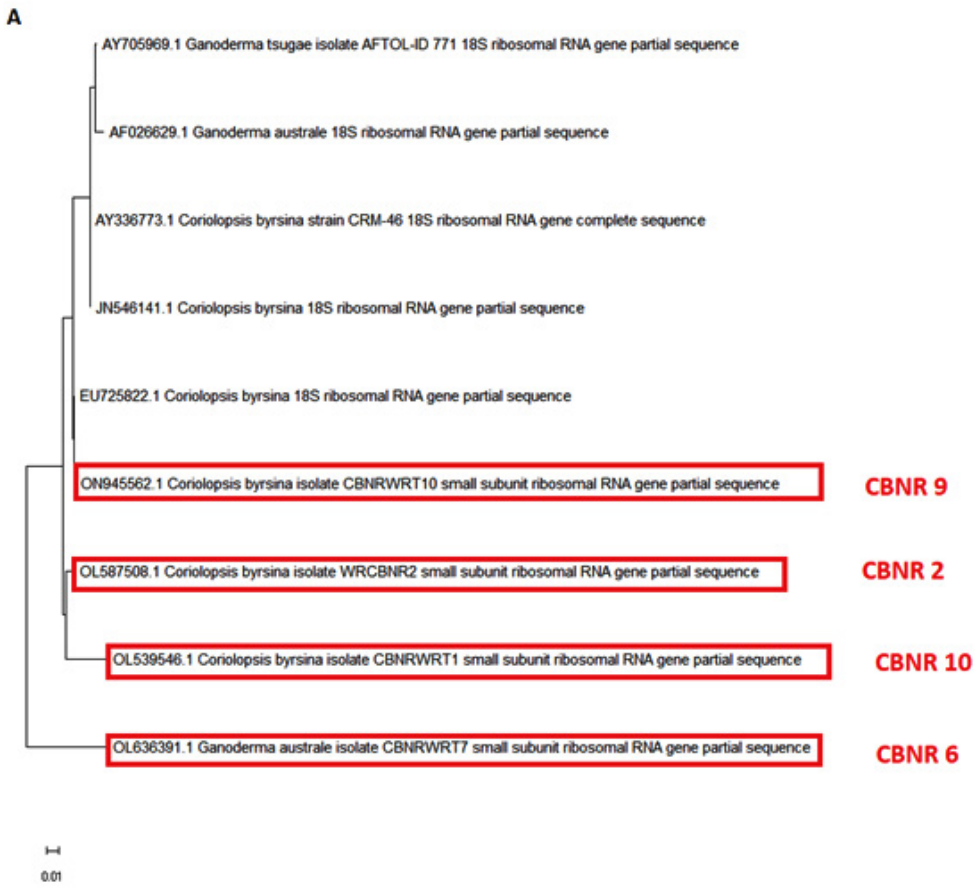
Molecular identification of fungal strains

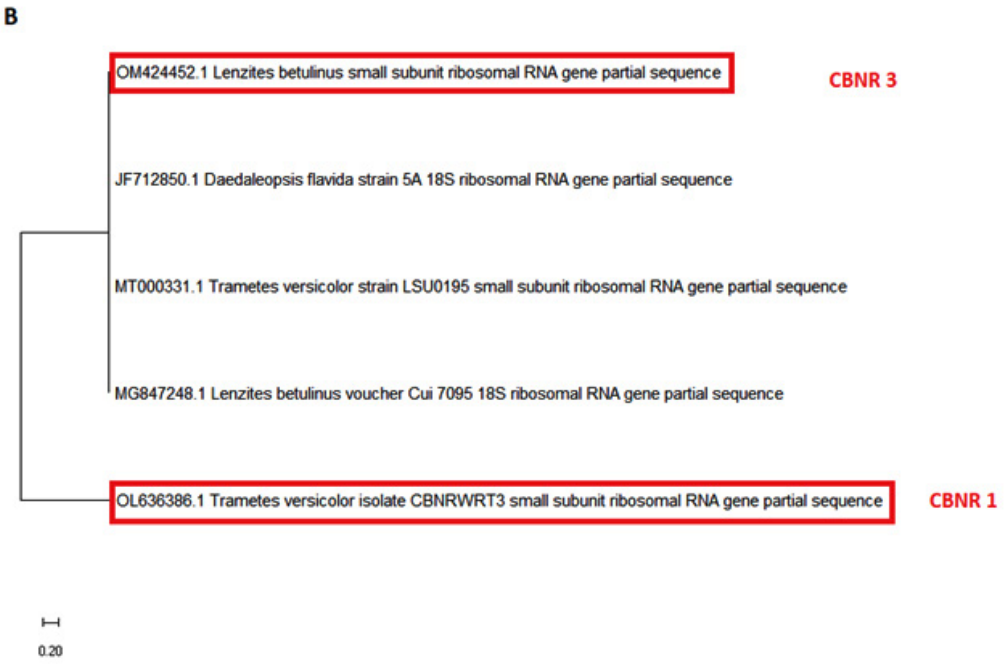
Genetic characterization using 18S rRNA has been considered as a sensitive tool to identify the fungal taxa. The Internal Transcribed Spacer (ITS) region is the suggested barcode for fungi since it has got good species resolution which covers broad spectrum of fungi. Banos et al. [23] describes the efficacy of using ITS region for identification of fungi in comparison to other marker genes. The molecular studies of lignin degrading white rot fungi have been reported in studies of Roseline et al. [29] and Badr El-Din et al. [11].

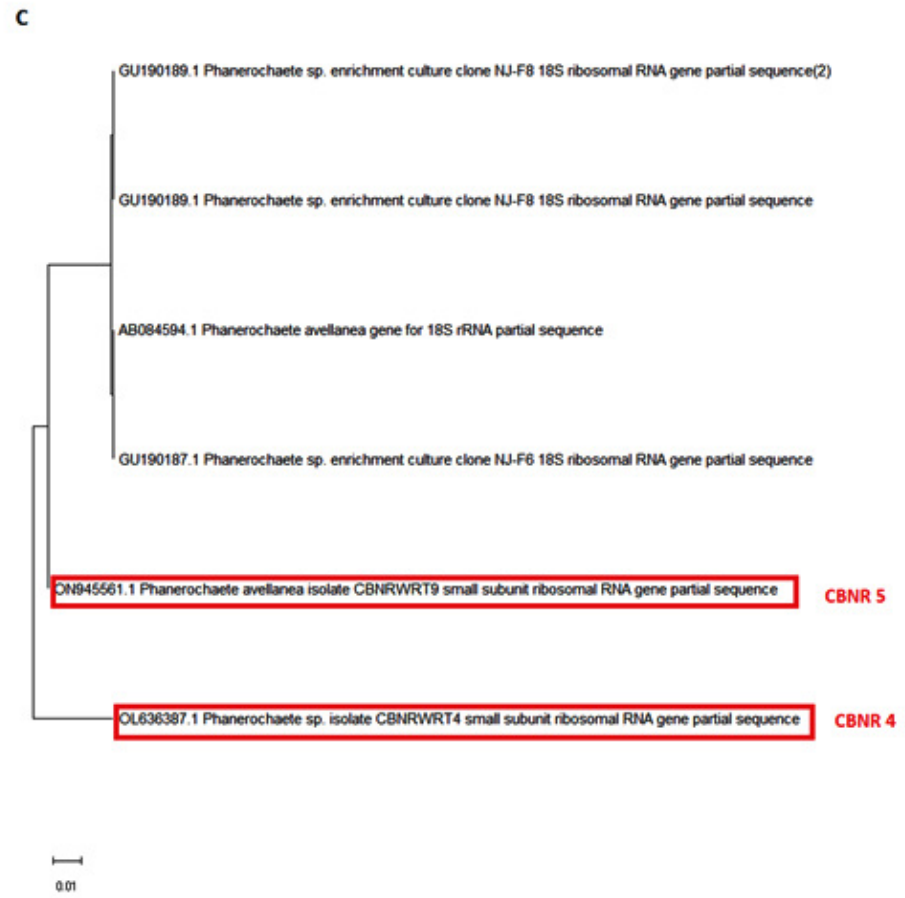
The strains having maximum homology were compared and aligned with ClustalW. The accession numbers obtained are OM424452, OL636391, OL636390, OL636388, OL636387, OL636386, OL587508, OL539546, ON945561 and ON945562. The dendrogram was constructed comparing fungal 18S rRNA gene sequences obtained with the similar sequences available in the NCBI Genbank.(figure 4 A,B,C,D)

Quantitative screening for Laccase, LiP and MnP

The isolates, after primary screening were further quantitatively analyzed for their ability to produce Laccase, Lip And MnP enzymes. Lignin modifying activity of white rot fungi are predominantly associated with the secretion of enzymes such as Laccase, lignin peroxidase and manganese peroxidase.




B
OM424452.1 Lenzites betulinus small subunit ribosomal RNA gene partial sequence
CBNR 3
JF712850.1 Daedaleopsis flavida strain 5A 18S ribosomal RNA gene partial sequence
MT000331.1 Trametes versicolor strain LSU0195 small subunit ribosomal RNA gene partial sequence
MG847248.1 Lenzites betulinus voucher Cui 7095 18S ribosomal RNA gene partial sequence
OL636386.1 Trametes versicolor isolate CBNRWRT3 small subunit ribosomal RNA gene partial sequence
CBNR 1
0.20


C
GU190189.1 Phanerochaete sp. enrichment culture clone NJ-F8 18S ribosomal RNA gene partial sequence(2)
GU190189.1 Phanerochaete sp. enrichment culture clone NJ-F8 18S ribosomal RNA gene partial sequence
AB084594.1 Phanerochaete avellanea gene for 18S rRNA partial sequence
GU190187.1 Phanerochaete sp. enrichment culture clone NJ-F6 18S ribosomal RNA gene partial sequence
ON945561.1 Phanerochaete avellanea isolate CBNRWRT9 small subunit ribosomal RNA gene partial sequence
CBNR 5
OL636387.1 Phanerochaete sp. isolate CBNRWRT4 small subunit ribosomal RNA gene partial sequence
CBNR 4
0.01

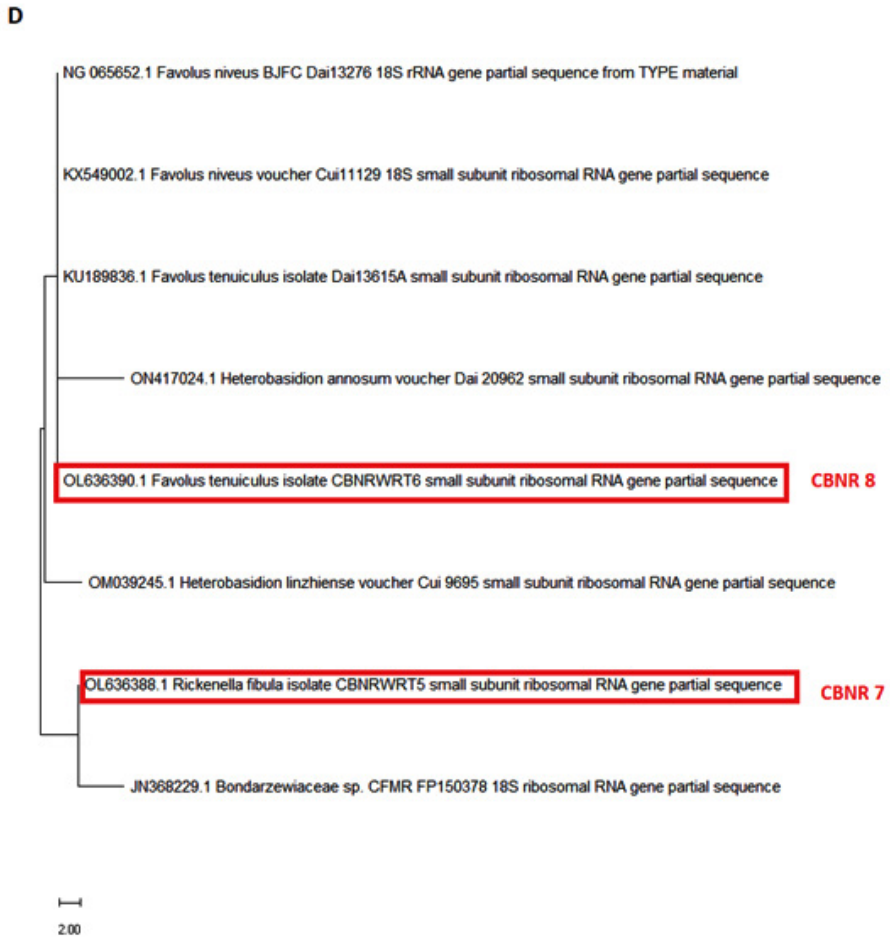


Fig. 4. Dendrogram showing 18s rRNA sequencing and sequence similarities of the isolates with related genus A. CBNR 2 *C.byrsina* , CBNR 6 *G.australe* , CBNR *9 C. byrsina* , CBNR 10 *C.byrsina;* B. CBNR 1 *T.versicolor* , CBNR 3 *L.betulina;* C. CBNR 4 *Phenerochaete*Sp. , CBNR 5 *P. avellanea*; D. CBNR 7 *R.fibula* , CBNR 8 *F. teniculus*

Enzyme production was carried out under static flask conditions using basal medium. Crude enzyme was filtered out and checked for the enzyme activity. In this study, all 10 strains exhibited lignolytic activities to varying degrees. Also, white rot fungi which are hardly exploited and studied for its lignolytic potentials have been identified. *Favolus teniculus*(OL636390), *Coriolopsis byrsina*(OL539546), *Lenzitus betulina*(OM424452) were positively responded to dye decolorization test and shown good Laccase, MnP and LiP activities on respective enzyme assays, suggesting they have lignin modifying abilities.

Trametes versicolor (Laccase activity 31.79 U/ml, MnP- 42.336 U/ml andLiP- 50.65), *Favolus teniculus* (Laccase activity 41.54 U/ml, MnP- 44.07 U/ml andLiP- 30.54 U/ml), *Coriolopsis byrsina*(Laccase activity 42.56 U/ml, MnP- 43.54 U/ml and LiP- 25.14) *Lenzitus betulinus* (Laccase activity 37.15U/ml, MnP- 38.97 U/ml &LiP - 30.43U/ml) were find to have promising lignolytic enzymatic system. (figure 5). Aydýnoðlu, [27] studied optimization of production of laccase from *Trametes versicolor* and reports laccase activity of 30.28 /g which is similar to our studies. *Yuliana* et al. [30] reports Laccase from *Trametes versicolor* with specific activity of 3.78

U/mg and purified enzyme shows the specific activity of 10.96 U/mg . However, the studies on enzymatic systems of other fungi *Favolus teniculus, Coriolopsis byrsina, Lenzitus betulinus* are scarce.

Laccase activity of these isolates ranged from 3.9U/ml to 45.75U/ml with the highest being produced by *Ganoderma austral*. Jing si et al. [31] reports the specific laccase activity of laccase enzyme isolated from *Ganoderma australe* as 22.214 U/mg and a putification fold of 23.989. MnP activity spanned 5.79 U/ml to 44.07 U/ml with the highest producer being *Favolus teniculus*. The enzyme activity of *Favolus teniculus* is being reported for the first time. Lignin peroxidase activity having a range of 13.1 U/ml to 50.65 U/ml where *Trametes versicolor* being the highest producer. Pooja singh et al. [32] studied the lignolytic enzyme activities of *Trametes versicolor* on oil palm biomass and reports the lignin peroxidase activity of 42.56 U/L.

While dye decolourization study, the isolates *Favolus teniculus*(OL636390), *Coriolopsis byrsina*(OL539546) *and Lenzitus betulina*(OM424452) were able to decolorize the dye methylene blue. This indicates the presence

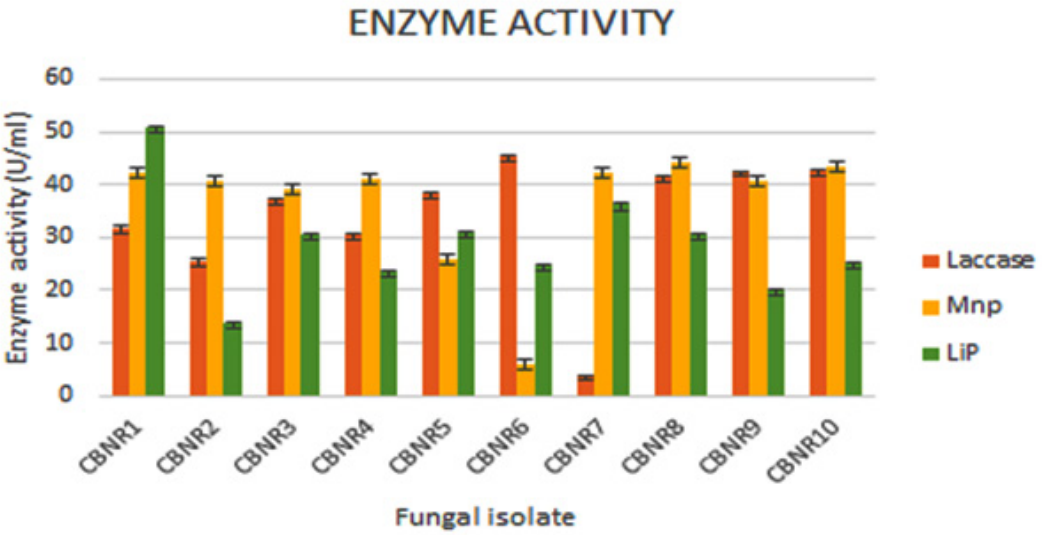


Fig. 5. Laccase, MnP and LiP enzyme activity of 10 isolates. Error bars represent the standard error of three replicates of experiment

Table 2. Molecular identification and screening of isolates for enzymatic activity

Isolate	Name of the strain	Primary screening		Enzyme assay(U/ml)		
		Methylene blue test	Guaiacol plate test	Laccase	LiP	MnP
CBNR 1	*Trametes versicolor* (OL636386)	+ve	+ve	31.37	50.65	42.33
CBNR 2	*Coriolopsis byrsina* (OL587502)	+ve	+ve	26.47	13.60	40.60
CBNR3	*Lenzitus betulinus* (OM424452*)*	+ve	+ve	37.46	30.43	38.97
CBNR 4	*PhenerochaeteSp* (OL636387)	+ve	+ve	30.16	23.55	41.09
CBNR 5	*Phenerochaete avellanea* (ON945561)	+ve	+ve	38.37	30.87	25.81
CBNR 6	*Ganoderma austral* (OL636391)	+ve	+ve	45.75	24.53	5.79
CBNR 7	*Rickenella fibula* (OL636388)	+ve	+ve	3.99	36.20	42.33
CBNR 8	*Favolus teniculus* (OL636390)	+ve	+ve	39.39	30.54	44.07
CBNR 9	*Coriolpsis byrsina* (ON945562)	+ve	+ve	42.53	20.07	40.73
CBNR 10	*Coriolopsis byrsina* (OL539546)	+ve	+ve	42.94	25.14	43.54

of extracellular oxidative enzymes produced by them, as reported by Dastidar et al[10].Similarly, all the three isolates were able to oxidize Guaiacol by forming a reddish brown zone indicating the presence of laccase enzyme produced by them. Further, quantitative enzyme assays has given a good picture regarding the lignolytic enzyme production by the three isolates. (Table 2).

Hataka et al.[6] studied lignolytic enzymes of white rot fungi and reported ligninolytic enzymes of *Phenerochete chryosporium*, *Cereporiopsis subvermispora*, *Pleurotus ostreatus*. Ramanaiah Illuri et al.[33] reports the lignolytic enzymes of basidiomycetes especially *Pleurotus sp.* Kirk et al.[5] detailed the mechanisms of enzyme production in *Phenerochete chryosporium*, *Cereporiopsis subvermispora*. The present result correlates with the previous reports and justifies the isolates have the potential to degrade lignocellulose effectively.

The potential industrial applications of white rot fungi in lignocellulosic waste management are numerous, and our study provides additional evidence of the effectiveness using such fungi to degrade lignin. Moreover, identifying new strains of white rot fungi could provide more sustainable and eco-friendly alternative to traditional chemical treatments for lignocellulosic waste. Optimizing the growth conditions of fungi through further investigations can enhance their lignolytic activity, thus leading the process efficient and cost-effective for industrial applications.

CONCLUSION

White rot fungi are the major group of basidiomycete that has adept capacity for efficient mineralization of lignin. Lignolytic enzymes of these white rot fungi have several industrial and biotechnological applications. In the present study 10 potential lignolytic enzyme producing White rot fungi were isolated, screened and molecular biologically identified using 18s RNA. Our study provides evidence of potential of white rot fungi as a sustainable alternative for lignocellulosic treatments. The isolation and screening of new strains such as those identified in our study *(Favolus teniculus*, *Coriolopsis byrsina and Lenzitus betulina*) could lead to the development of more effective and efficient biotechnological systems for various industrial applications.

Conflict of Interest

There is no conflict of interest.

Funding Sources

There is no funding sources.

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