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RESEARCH ARTICLE

SELECTION AND SCREENING OF HIGHEST LIGNOLYTIC WHITE ROT FUNGUS FROM WARANGAL DISTRICT AND ITS MOLECULAR IDENTIFICATION

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ABSTRACT

Hundred isolates of white-rot fungi were tested for their efficiency of wood degradation based on guaiacol-oxidation, lignin peroxidase, laccase and manganese peroxidase activities. The purpose of the test was to select the best isolates that show high efficiency of producing lignolytic enzymes. Among these isolate BR-1 (*Pycnoporus cinnabarinus*) was identified as the most good and highest activity for lignin degradation. Further its identification was confirmed in molecular level carried by the internal transcribed spacer 'ITS' regions of the ribosomal DNA (28S rDNA) sequencing. The D2 region of LSU (Large subunit 28S rDNA) gene sequence was used to carry out BLAST with the database of NCBI gene bank database.

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INTRODUCTION

Lignin is a heterogeneous, branched and complex polymer consisting of phenylalanine-derived aromatic subunits (Whetten and Sederoff, 1995). Because of its recalcitrance, lignin complicates the utilization of biomass polysaccharides in biorefineries and increases the energy consumption in mechanical pulping (Jiang *et al*., 2008a). In nature one group of organisms, the basidiomycetous fungi are able to effectively degrade lignin by employing a family of lignin degrading enzymes. Its degradation plays a key role in the carbon cycle of the biosphere Tein (1987). Previous research done revealed that the white-rot basidiomycetes degrade lignin more extensively and rapidly than any other known group of organism (Cullen, 1996; Singh and Chen, 2000; Akhtar *et al.,* 1997). In contrast to other fungi and bacteria, white-rot fungi are capable of completely degrades lignin to carbon dioxide and water. *P. coccineus* and *C. versicolor,* are white-rot basidiomycetes that are distributed worldwide. They have been studied in this current research for the production of ligninolytic enzymes. Laccases, peroxidases (including lignin peroxidases, manganese peroxidases and manganese-independent peroxidases) and H_2O_2-generating oxidases are components of the lignin-degrading enzyme system (Hatakka, 1994). There has been great interest in using fungal laccases and peroxidases for biotechnological processes due to their chemical and catalytic features (Messner and Srebotnik 1994; Sayadi and Ellouz 1995; Ferraz *et al*., 2008). The aim of this research work is to screen the highest ligninolytic enzymes; peroxidase, manganese peroxidase, laccase, and cellulase producing white rot fungi from Warangal district and its molecular identification

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MATERIALS AND METHODS

Collection of fungi

White rot fungi in the form of fruiting bodies were collected from forests in Warangal districts, Telangana state. They were placed into plastic bags and cleaned with disinfectants and kept into test tubes containing fresh MEA (Malt Extract Agar Medium). The samples were marked with information such as number, procurement location, growth site and specific characteristics.

Isolation of fungi

White rot fungi were isolated using bait made of malt extract agar for the culture medium. Vegetation such as twigs,

branches and leaves showing signs of attack by fungi was cut-off, cleaned with disinfectants, rinsed with distilled water and then placed on agar medium in petri-dishes. In addition, fruiting bodies of fungi were cleaned with disinfectants and approximately 3 x 3 mm was placed on agar medium in petri-dishes. Later on, when the mycelium had grown on the medium in the vicinity of the tissues, the sample was transferred to fresh agar medium tubes. This was repeatedly carried out until pure cultures could be obtained as single cultures or so-called fungal isolates.

Selection of white-rot fungi for lignolytic activity

The selected twenty three white-rot fungi were conducted by the methods of Nishida *et al*. (1988). Isolates were inoculated in wood-agar medium containing guaiacol and then incubated at room temperature for 7 days. Observations were carried out by measuring the diameter of ring-shaped mycelia.

Enzyme assays

White rot fungi were grown in malt extract broth, incubated for 14 days. Activities of enzymes laccase (E.C.1.10.3.2), lignin peroxidase (E.C.1.11.1.14) and manganese peroxidase (E.C. 1.11.1.13) were measured using guaiacol as substrate at the end of 7 and 14 days of incubation. For three enzymes, one unit of enzyme was defined as the amount of enzyme necessary to oxidize one µ mol of substrate per minute

DNA extraction

DNA was extracted from pure culture of selected white rot fungi. Approximately 2 cm^2 of mycelium was collected and introduced into an eppendorf tube containing 1 ml of distilled water and 12 to 15 glass beads with a diameter of 3 mm (Merck). After vortexing at maximum speed for 2 min, 100 µl of a suspension containing fragmented mycelium was transferred to a second tube containing an equal volume of <106-µm glass beads (Sigma). The mycelium was further disrupted for 5 min by shaking in a disintegrator followed by three subsequent steps of freezing in liquid nitrogen and heating at 95 °C for 5 min. After centrifugation, 35 µl of supernatant was mixed with 150 µl of 100% ethanol and loaded onto an QIAamp DNA mini kit column (Qiagen, Basel, Switzerland). The DNA was purified by following the protocol provided by the manufacturer and eluted with 200 µl of distilled water.

PCR and Sequencing

Universal primers ITS1 (5'ACCCCGCTGAACTTAAGG-3') ITS4 universal (5'GGTCCGTGTTTCAAGACGG3') [23] were procured from Bangalore. DNA amplifications were performed in a model PTC 200 thermal cycler. PCR was performed in a 25 µl reaction mixture with final concentration (per reaction) of 1PCR core buffer, 2.5 mM MgCl2, 0.2 mM (each) dNTPs, 10 pmol of each primer, 1 U of AmpliTaq and 50 ng of template. First denaturation was carried out for 3 min at 95 °C. Initial amplifications were performed as 5 cycles of 95 °C for 30 s (denaturation), 52 °C for 30 s (annealing) and 72 °C for 1.5 min (extension). Further amplification was performed as 25 cycles of 95 °C for 30 s (denaturation), 51 °C for 30 s (annealing) and 72 °C for 1.5 min (extension), followed by a 10 min final extension of 72 °C. Negative controls were carried out without template to ensure there was no contamination. PCR products were resolved by electrophoresis in 2% low melting point agarose, and visualized by ethidium bromide staining. Sequencing of the PCR products was carried out by on an automated multi-capillary DNA sequencer, ABI Prism 3130xl genetic analyzer using the big dye terminator v.3.1 ready reaction BDT v3.1 cycle sequencing kit. Sequences were then matched with those already known using the BLAST (Basic local alignment search tool) search option at NCBI GenBank (http://www.ncbi.nlm.nih.gov) and based on maximum identity score first ten sequences were selected for construction of the phylogenetic tree.

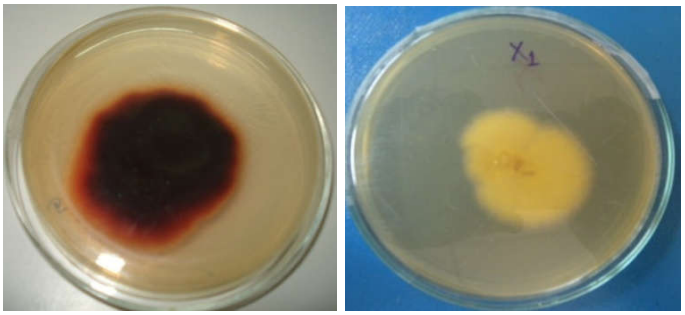
RESULTS AND DISCUSSION

The efficiency of white rot fungi in lignolytic activity, a detailed screening was under taken in various habitats of Warangal district and collected the fungi. They were cultured, purified, and screened based on their lignolytic potentials. The obtained data is presented in Table 1-3. From the Table 1 it was noted that the white rot fungi collected showed different ranges of enzyme activity in qualitative basis by guaiacol plate method. Among 100 cultures only fifty-eight (58) cultures showed the guaiacol digestion with the range between 5 (BR-32) to 58 mm (BR-73) and 42 cultures failed to produce any lignolytic activity. Among the 58 strains the range of lignolytic activity was 1 -10 mm in two; 11-20 mm in sixteen; 41-50 mm in seven and 51- 60 mm in three strains. Above 50% of strains recorded the moderate lignolytic enzyme activity whiles less than 5% strains secreted the enzyme in meager quantities. The strains BR-1, BR- 52 and BR- 73 are considered to be potential in secretion of this enzyme as their digestion zone was above 50 mm. The strains BR-9, BR-19, BR-21, BR-60, BR-61, BR-62, and BR-63 showed their lignolytic activity in moderate to high range (41-50 mm). The remaining 43 strains showed their range of activity is less to moderate levels (11- 40 mm).

The quantitative assay of three lignolytic enzymes viz., laccase, lignin peroxidase and manganese peroxidase in one hundred white rot fungal cultures was assayed in submerged state during their 7 and 14 days of incubations and the data collected was presented in Table 2. From the Table 2 it was evident that there was a significant correlation between qualitative assay of the cultures and with quantitative secretion of the three lignolytic enzymes. The strain which are responsible for maximum amount of guaiacol digestion were showed highest amount of laccase and ligninperoxidase. However, the enzyme, manganese peroxidase did not respond in its production with qualitative assay. The maximum laccase was recorded in BR-1 (260 U/ml), BR-9 (204 U/ml), BR-19 (252 U/ml), BR-21 (244 U/ml), BR-30 (236 U/ml), BR-44 (210 U/ml), BR-73 (245 U/ml). The secretion was high in 7 days old cultures over 14 days and there was substantial reduction in the amount of laccase secretion in 14 days cultures. The following strains produced moderate amount of laccase BR-12 (160 U/ml), BR-51 (125 U/ml), BR-61 (128U/ml), BR-63 (145 U/ml), BR-74 (132 U/ml).

The cultures which are responsible for the production of less to moderate amounts are BR-4 (66 U/ml), BR-15 (52 U/ml), BR-20 (48 U/ml), BR-29 (56 U/ml), BR-33 (40 U/ml), BR-37 (48 U/ml, BR-45 (86 U/ml), BR-46 (82 U/ml), BR-47 (80 U/ml), BR-49 (68 U/ml),BR -59 (84 U/ml), BR- 63 (45 U/ml), BR-67 (44 U/ml), BR-69 (42 U/ml), BR-70 (52 U/ml), BR -75 (40 U/ml), BR-83 (40 U/ml). The remaining cultures produced very meager amount of laccase i.e. Less than 40 U/ml. Except one culture (BR-88) all the other cultures were produced laccase in different quantities. BR-91 failed to produce laccase in 7 days incubation but secreted meager amounts (4 U/ml) in 14 days of incubation. The second best lignolytic enzyme secreted by majority of white rot fungal cultures is lignin peroxidase. Though it is varied in its production among 100 cultures, it synchronized with the qualitative assay. The maximum producers of lignin peroxidase are – BR-1 (92 U/ml), BR-19 (64U/ml), BR-21 (74 U/ml), BR-25 (54 U/ml), BR-29 (60 U/ml), BR-49 (48 U/ml), BR-52 (60 U/ml), BR-73 (48 U/ml).

The moderate quantities of lignin peroxidase was secreted by the following white rot fungal strains BR-4 (28 U/ml), BR-5 (24 U/ml), BR-7 (24 U/ml), BR-11 (24 U/ml), BR-13 (24 U/ml), BR-20 (40 U/ml), BR-37 (32 U/ml), BR-48 (24 U/ml), BR-58 (28 U/ml), BR-63 (26 U/ml), BR-75 (32 U/ml), BR-83 (22 U/ml) and BR-99 (20 U/ml) on 7 days on incubation (Table 2). The remaining cultures are very meager producers of lignin peroxidase and the quantities are less than 20 U/ml. As in laccase production, lignin peroxidase secretions were also less during 14 days of incubation. Majority of white rot fungal cultures failed to secrete manganese peroxidase enzyme. Among the producers, the maximum secretors are, BR-1 (40 U/ml), BR-63 (44 U/ml), and BR-69 (20 U/ml). The meager to moderate quantities of this enzyme was recorded is BR-15 (18 U/ml), BR-14 (16 U/ml), BR-21 (14 U/ml), BR-25 (16 U/ml), BR-59 (16 U/ml) and BR-75 (18 U/ml) on 7 days of incubation. Among the remaining strains some produced very meager amounts and some strains completely failed to secrete it. Based on the abilities of the production of the lignolytic enzyme, the cultures were screened for their applications in wood based industries.



Positive Plate **Negative Plate**

Figure 1. Positive and negative results of white rot fungi for lignolytic enzymes by guaiacol plate technique

Research was initiated by screening of white rot fungi for selective removal of lignin from wood blocks (Otjen *et al.*, 1987; Blanchette *et al.*, 1997). Wide variations were found among species and among strains within certain species. A faster method for screening for selective lignin removal was described by Nishida *et al.* (1988) and this method is based on the formation of colour during growth of test strains of guaiacol -wood meal agar plates (Fig. 1). The bio pulping and bleaching consortium reported on a more targeted procedures based on the effect of fungal treatment of coarse pulp on pulp trainability (Leatham and Mycers, 1990). Lund and Ragauskas (2001), Chandra and Ragauskas (2002), Camarero *et al.*, (2004) Rodriguez and Toca (2006) reported that lignolytic enzymes are able to polymerise lignin and delignify wood pulps, kraft pulp fibers for the manufacture of paper. There has been a growing environmental concern about chlorinated organic substances including toxic, mutagenic, carcinogenic, dibenzofurens and phenols in the effluent from the bleaching and conventional kraft pulp (Alder *et al.*, 2009). Fungal treatment helps to decrease the negative environmental impact of pulp and paper production.

Table 1. Qualitative assay of lignolytic enzymes by guaiacol plate method in one hundred white rot fungal cultures

Fungi	Guaiacol digestion zone (in mm)	Fungi	Guaiacol digestion zone (in mm)
BR -1	55	BR-51	35
BR -2	-	BR-52	55
BR-3	-	BR-53	-
BR-4	25	BR-54	12
BR-5	-	BR-55	40
BR-6	-	BR-56	38
BR-7	18	BR-57	35
BR-8	-	BR-58	53
BR-9	45	BR-59	20
BR-10	12	BR-60	50
BR-11	35	BR-61	45
BR-12	40	BR-62	45
BR-13	30	BR-63	46
BR-14	13	BR-64	-
BR-15	24	BR-65	27
BR-16	-	BR-66	13
BR-17	-	BR-67	16
BR-18	15	BR-68	-
BR-19	45	BR-69	30
BR-20	30	BR-70	35
BR-21	46	BR-71	-
BR-22	16	BR-72	22
BR-23	-	BR-73	58
BR-24	20	BR-74	38
BR-25	19	BR-75	21
BR-26	-	BR-76	35
BR-27	10	BR-77	30
BR-28	26	BR-78	20
BR-29	24	BR-79	14
BR-30	44	BR-80	12
BR-31	-	BR-81	-
BR-32	5	BR-82	-
BR-33	12	BR-83	-
BR-34	-	BR-84	-
BR-35	26	BR-85	-
BR-36	22	BR-86	-
BR-37	16	BR-87	-
BR-38	-	BR-88	-
BR-39	-	BR-89	-
BR-40	-	BR-90	
BR-41	25	BR-91	-
BR-42	22	BR-92	-
BR-43	37	BR-93	5
BR-44	40	BR-94	8
BR-45	35	BR-95	10
BR-46	33	BR-96	10
BR-47	24	BR-97	-
BR-48	-	BR-98	-
BR-49	34	BR-99	-
BR-50	40	BR-100	-

Table 2. Quantitative assay of three Lignolytic enzymes in one hundred white rot fungal cultures

	Lignolytic enzymes (U/ml)					
	Laccase		Lignin Peroxidase		Manganese Peroxidase	
	7 Days	14Days	7Days	14 Days	7 Days	14 Days
BR -1	260	68	92	32	40	12
BR -2	8	20	16	12	-	-
BR-3	12	8	6	12	-	-
BR-4	66	32	28	36	20	-
BR-5	8	2	24	4	12	-
BR-6	24	4	8	12	12	-
BR-7	12	4	24	4	12	-
BR-8	16	12	4	16	4	-
BR-9	254	34	12	20	4	-
BR-10	28	16	16	4	4	-
BR-11	28	20	24	16	12	-
BR-12	160	32	14	8	-	-
BR-13	8	16	24	12	4	-
BR-14	16	4	8	4	16	-
BR-15	52	32	48	16	18	4
BR-16	24	12	16	8	12	-
BR-17	12	16	8	-	-	-
BR-18	16	8	8	8	2	-
BR-19	252	16	64	12	-	-
BR-20	48	40	40	42	-	8
BR-21	244	46	74	8	14	4
BR-22	4	8	12	8	12	-
BR-23	12	12	4	8	-	8
BR-24	14	4	24	4	-	4
BR-25	52	20	54	10	16	4
BR-26	22	16	6	4	6	-
BR-27	38	18	4	12	8	-
BR-28	12	12	4	-	12	-
BR-29	56	30	60	32	10	6
BR-30	236	70	18	8	12	10
BR-31	4	8	16	16	-	4
BR-32	4	16	12	8	8	-
BR-33	40	10	15	5	5	-
BR-34	12	4	8	4	4	-
BR-35	20	40	16	16	-	-
BR-36	24	12	12	4	-	4
BR-37	48	40	32	20	8	8
BR-38	22	12	16	12	-	-
BR-39	24	10	15	8	6	-
BR-40	2	6	16	8		
BR-41	12	4	20	-	-	-
BR-42	16	6	24	4	-	-
BR-43	20	8	4	12	-	-
BR-44	210	42	8	20	-	-
BR-45	86	12	12	10	-	-
BR-46	82	20	36	12	-	-
BR-47	80	28	4	12	-	-
BR-48	32	4	24	4	-	-
BR-49	68	24	48	36	8	16
BR-50	8	4	8	4	-	-
BR-51	125	12	14	6	8	2
BR-52	208	45	60	18	-	-
BR-53	8	4	4	2	-	-
BR-54	6	4	16	2	-	-
BR-55	12	4	16	8	12	-
BR-56	8	16	12	6	4	-
BR-57	20	12	10	4	4	-
BR-58	4	12	28	12	-	-
BR-59	84	36	-	10	16	7
BR-60	212	20	12	30	2	
BR-61	128	18	8	8	12	8
BR-62	116	8	12	8	4	-
BR-63	45	56	26	8	44	4
BR-64	8	8	12	4	12	-
BR-65	16	8	8	4	4	-
BR-66	28	12	20	18	12	4
BR-67	44	32	10	8	4	-
BR-68	8	8	4	12	-	8
BR-69	42	32	20	12	20	8
BR-70	52	28	4	6	4	4

……*Continue*

BR-71	18	4	4	4	8	-
BR-72	12	8	4	12	12	4
BR-73	245	90	78	34	4	4
BR-74	132	30	16	12	12	-
BR-75	40	32	32	16	18	4
BR-76	4	16	12	4	12	4
BR-77	12	8	8	6	-	-
BR-78	8	12	20	6	-	6
BR-79	4	4	4	12	-	-
BR-80	6	12	4	2	-	-
BR-81	10	8	4	4	-	-
BR-82	4	4	4	4	-	-
BR-83	40	14	22	10	12	-
BR-84	28	12	12	4	-	-
BR-85	24	16	12	20	-	-
BR-86	12	8	16	16	-	-
BR-87	14	6	12	4	4	-
BR-88	-	-	4	8	--	-
BR-89	16	4	8	4	8	-
BR-90	8	12	16	8	4	-
BR-91	-	4	4	-	-	-
BR-92	24	16	12	12	-	8
BR-93	20	12	8	12	-	-
BR-94	16	4	12	4	-	-
BR-95	12	5	15	8	-	-
BR-96	32	12	12	-	-	-
BR-97	12	6	12	6	8	-
BR-98	28	20	16	4	-	-
BR-99	12	40	20	16	-	4
BR-100	32	24	16	16	-	-

Table 3. List of selected white rot fungal cultures with potential lignolytic activity

Sl.No	Selected Fruit bodies
1	BR-1
2	BR-4
3	BR-9
4	BR-12
5	BR-19
6	BR-21
7	BR-30
8	BR-44
9	BR-45
10	BR-46
11	BR-47
12	7BR-49
13	BR-51
14	BR-52
15	BR-59
16	BR-60
17	BR-61
18	BR-63
19	BR-73
20	BR-74

Removal of lignin from wood is the first step in the manufacturing of chemical paper pulps and the most common process Veronica *et al.* (2010). Examined that kraft pulp was bleached in a totally chlorine free sequence that involved treatments with culture supernatants from white rot fungus, *Tramates troggi* followed by a peroxidase stage. Ahmad *et al.* (2011) studied the efficiency of *Pycnoporus coccineus* and *Coriolus versicolor* on the production of lignolytic enzymes during biopuling of *Acacia mangium* wood chips and noticed that lignin content was significantly decreased in wood chips bio treated with *Coriolus versicolor* (9.42%) compared to *Pycnoporus coccineus* (8-10%). Singh *et al.* (2013) screened diversified white rot fungal strains for bio pulping and bio bleaching in wood based industries for manufacture of paper and their enzyme profiles under different modes of cultivation. They found that that the indigenenously isolated white rot fungi exhibited variable xylanase, laccase, cellulase activities. Among the screened fungi two isolates produced good xylanase and laccase activities and minimal cellulase activity, there by indicating that these two white rot fungal strains can very well be tested as pulp bio bleaching after 7 days of incubation. Badr El-Din *et al.* (2013). Among 62 screened fungal isolates for lignin peroxidase production, the most potent isolates for lignin peroxidase production were identified using the DNA sequence of the internal transcribed spacer (ITS) region as *Phenerochaete chrysosporium* and *Pleurotus ostreatus*. They further reported for the pretreatment of rice straw with *Pleurotus ostreatus* caused moderate pulp yield losses (5.8%) and preferential lignin degradation (Kappa number losses of 34.6%). This indicates that *Pleurotus ostreatus* is superior for use in the bio pulping process. Recently, Pandy and Albert (2014) studied the compatibility of lignolytic white rot fungi with *Trichoderma reesei* for practical applications of co-culture in bio pulping and bio bleaching of pulp in paper industry.

The activities of lignin peroxides, laccase and Manganese peroxidase were assayed for all the 100 strains. Among them 20 strains were more promising (Table 3). BR-1 showed the maximum activity of lignin peroxides, laccase and manganese peroxidase. Hence, BR-1 was selected for molecular characterization for further confirmation of genus and species. The genomic DNA of strain BR-1 was subjected to PCR-amplification of the ITS region by using ITS1 and ITS4 primers. The amplified PCR product turned out to be approximately 700 bp in length as shown in the Fig. 2. DNA sequencing: The amplicon DNA was gone to the sequencing using DF (direct forward) DF: 5'ACCCCGCTGAA CTTAAGG-3' and (direct reverse) DR: 5'GGTCCGTGTTTC AAGACGG3' Primers using BDT v3.1

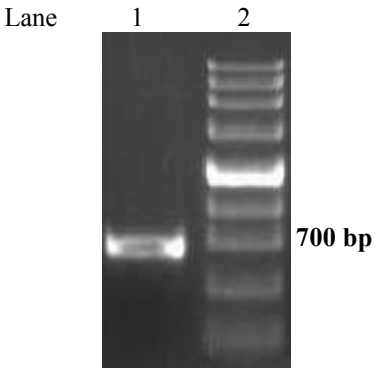


Figure 2. Gel Image of PCR amplicon
Lane 1 – DNA amplicon, Lane 2 – DNA Marker

Cycle sequencing kit on ABI 3730 xl Genetic Analyzer. Comparison and constructing the phylogenetic tree: D2 region of LSU seq was compared with database using the BLAST (Basic Local Alignment Search Tool) search tool, based on maximum identity. Identities were taken and constructed the phylogenetic tree. The closest sequence was *Pycnoporous cinnabarinus* (Fig. 3).

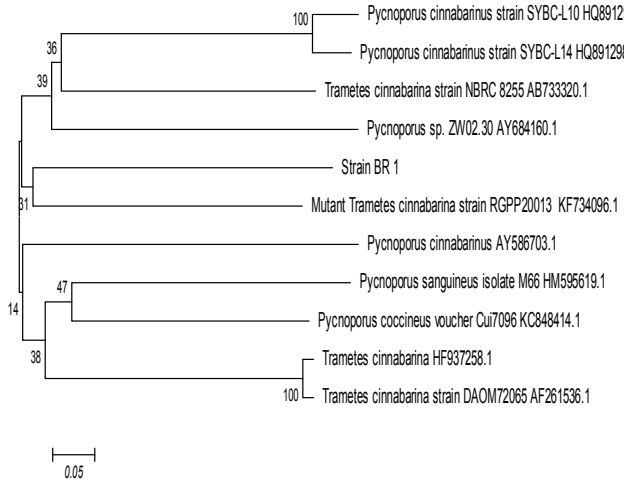


Figure 3. Phylogenetic tree

Conclusion

From the present investigations it is revealed that among 100 strains of white rot fungi screened for lignolytic enzymes, strain BR-1 found to be most promising. Based on molecular characterization this strain was further confirmed as *Pycnoporous cinnabarinus.* Further detailed investigations are needed for the optimization of lignolytic enzymes.

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