Journal of Cell and Tissue Research Vol. 11(1) 2557 - 2562 (2011)
ISSN: 0974 - 0910 *(Available online at www.tcrjournals.com)* **Original Article**

SELECTION OF HIGHEST LIGNOLYTIC WHITE ROT FUNGUS AND ITS MOLECULAR IDENTIFICATION

METUKU, R. P.[?], BURRA, S., NIDADAVOLU, S.V. S. S. S. L. HIMA BINDU, PABBA, S. AND MA. SINGARACHARYA

Department of Microbiology, Kakatiya University, Warangal, 506 009, A.P.
E. mail: metuku.ram@gmail.com

Received: December 21, 2010; Accepted: January 24, 2011

Abstract: Twenty three 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 ligninolytic enzymes. Among these KU1009 (Pycnoporus sp. DIS 343f) strain was identified as the most superior for lignin degradation. Further its identification 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. Based on maximum identity score first ten sequences were selected, phylogenetic tree was constructed using MEGA 4.

Key words: White rot fungi, Ligninolytic enzymes

INTRODUCTION

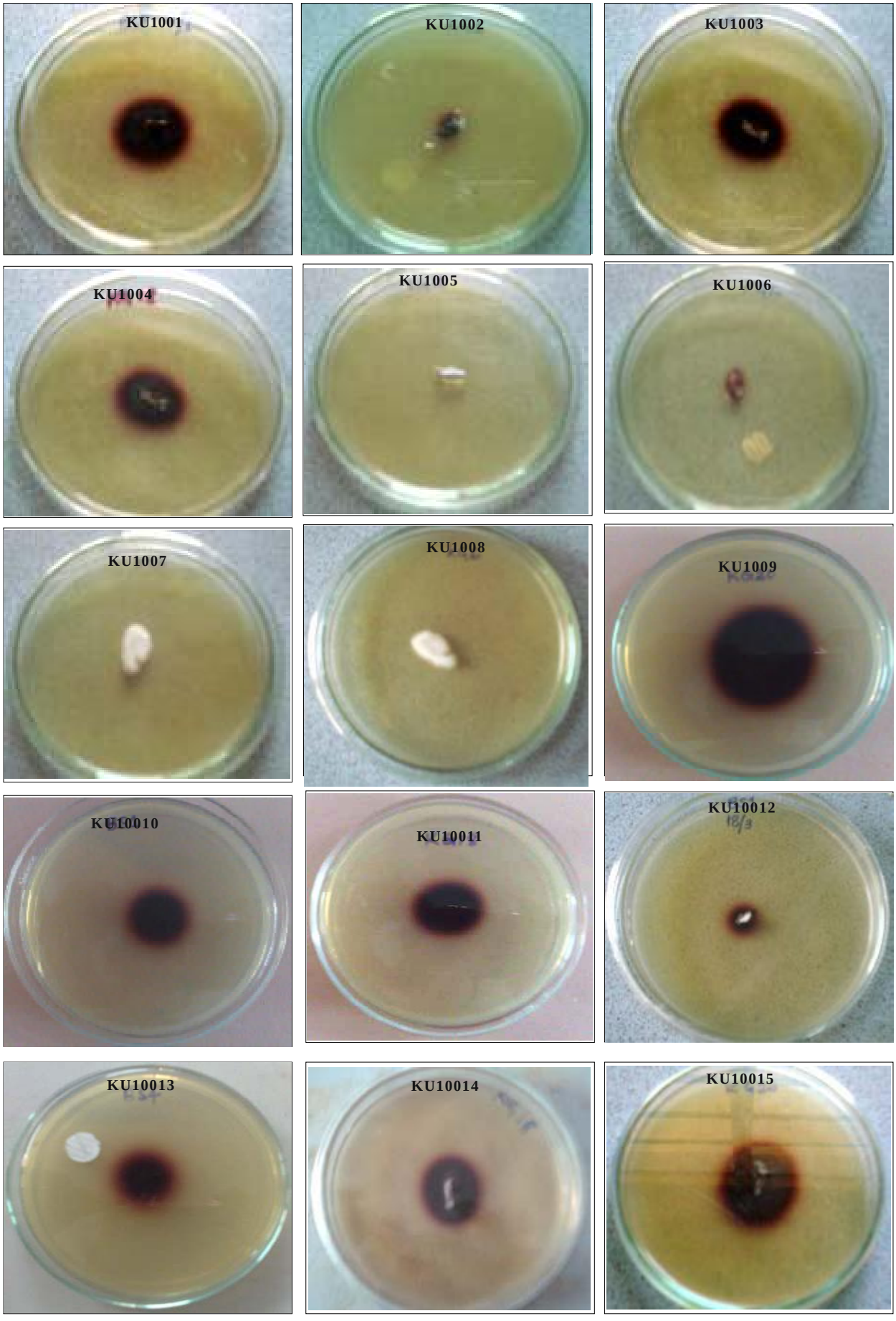
White rot fungi have been demonstrated to be good candidates for xenobiotic decontamination through biotechnological processes. These ligninolytic organisms are able to metabolize chemically diverse molecules, including chlorinated phenols, pesticides, PCBs, dioxins, organophosphorus compounds, nitrotoluenes, chloranilines, dyes, and other compounds of environmental concern[1-3]. Their extra cellular enzymatic system, which is involved in lignin degradation, consist mainly of oxidative enzymes: laccases, lignin peroxidases (LiP) and manganese peroxidases (MnP).

Lignin is a structurally complex aromatic biopolymer which is recalcitrant to degradation. This is an important step in the mineralization of carbon in nature. It has been demonstrated that the extra cellular enzymes of some white rot fungi are able to degrade lignin extensively. In addition to their industrial application in delignification, these peroxidases are able to oxidize a wide range of substrates with high redox potential [4]. Both peroxidases and laccases catalyze substrate oxidation by a mechanism involving free radicals, and showing low substrate specificity. These ligninolytic enzymes are directly involved in the degradation of various xenobiotic compounds, including industrial dyes [5-7], polycyclic aromatic hydrocarbons [8,9], pesticides [10,11], dioxins [12,13], chlorophenols [14,15], explosives [16,17] and kraft pulp bleaching [18].

MATERIALS AND METHODS

Collection of fungi: Fungi in the form of fruiting bodies were collected from forests in Andhra Pradesh. They were placed into plastic bags and cut, cleaned with disinfectants and put into 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: Fungi were isolated using bait made of malt extract agar for the culture medium.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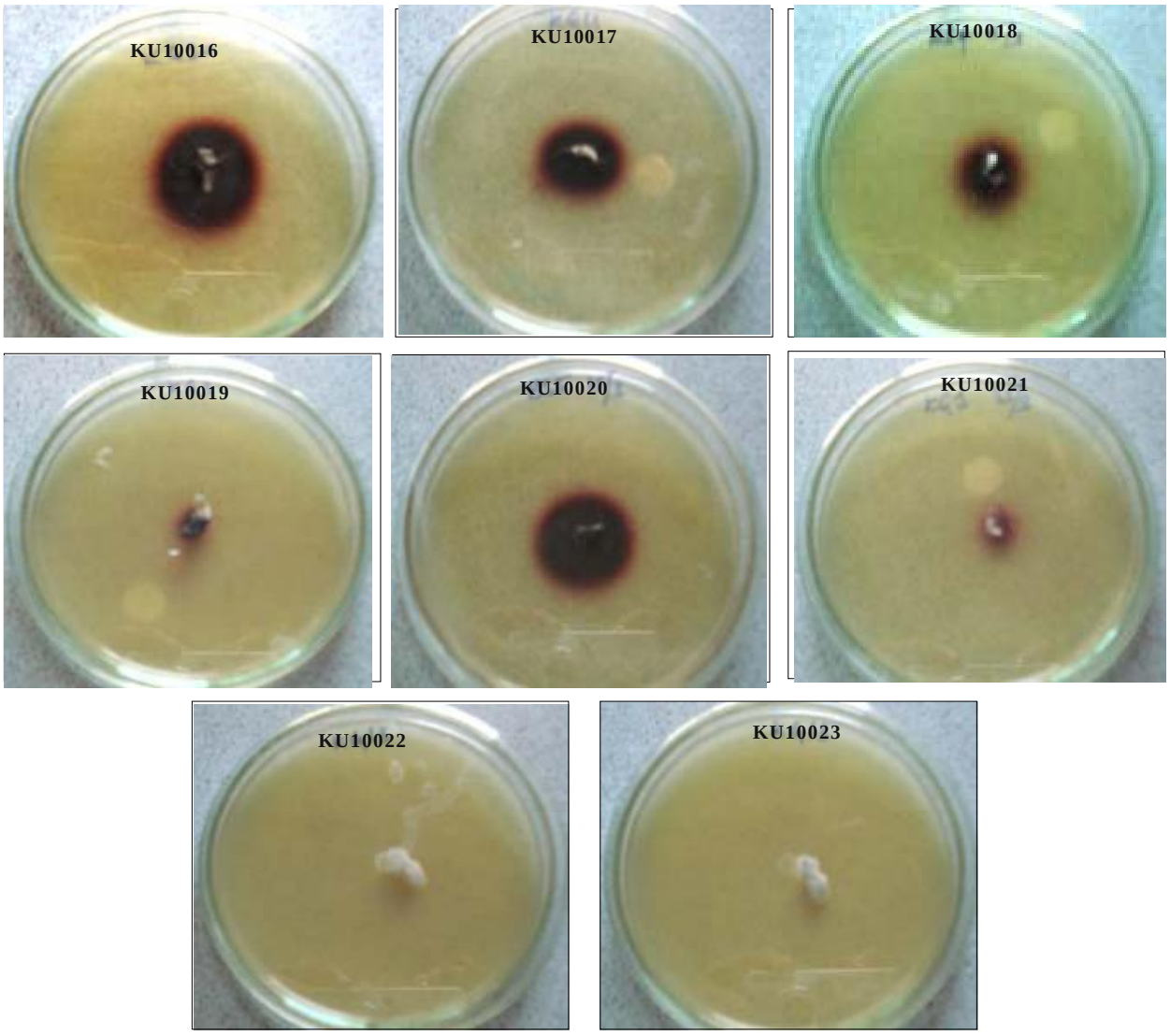

Fig. 1: The figures KU1001 to KU1023 shows the guaiacol oxidation plates by different white rot fungi. Note that KU1009 showed the highest zone of oxidation.

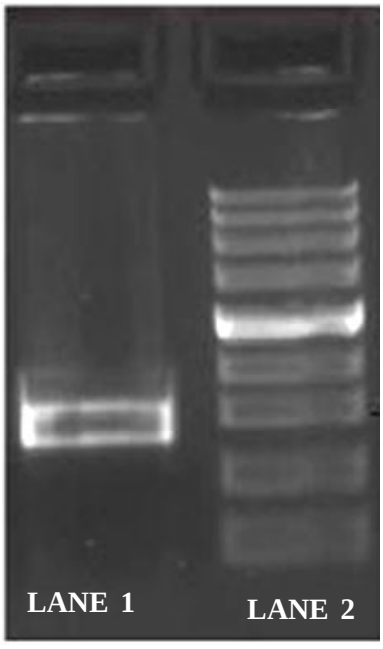

Fig. 2: The Gel electrophoresis showing the result of amplified PCR product of 700bps. Lane 1 D2 region of LSU Lane 2 DNA markers

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 media in 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. [19]. 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 7 days. Activities of enzymes laccase (E.C.1.10.3.2) [20] lignin peroxidase (E.C.1.11.1.14) [21] and manganese peroxidase (E.C. 1.11.1.13) [22] were measured using guaiacol as substrate. For there three enzymes, one activity unit was defined as the amount of enzyme necessary to oxidize one µ mol of substrate per minute

DNA extraction: DNA was extracted from pure cultures on malt extract agar medium. 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 a 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 $MgCl_2$, 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 using MEGA4 [24].

RESULTS

Genomic DNA was successfully isolated from the mycelia of *KU1009*. The results shows high diameter zone (Fig. 1). Table 1 shows guaiacol oxidation in mm and enzyme activities in U/ml.

Enzyme assay: The activities of lignin peroxides, laccase and Manganese peroxidase were assayed for all the strains, but *KU1009* showed the highest activity of lignin peroxides (576 U/ml) and laccase (486 U/ml) and moderate activity of manganese peroxidase (60 U/ml).

The genomic DNA of KU1009 were 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'ACCCCGCTGAACTTAAGG-3' and (direct reverse) DR: 5'GGTCCGTGTTTCAAGACGG3' Primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl 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, E value; the first ten (DQ327661.1, AY855912.1, AY351921, FJ372693.1, AF261536.1, AY586703.1, J488128, AY684160.1, AF518625.1, AF393074.1). Identities were taken and constructed the phylogenetic tree using MEGA 4 software .The closest sequence was Pycnoporous sp. DIS 343f was conformed as show in the table 2.

Table 1: The table shows the diameter zone of Guaiacol oxidation of each plate in mm and lignin peroxidase, laccase and manganese peroxidase activities in units/ml

S.No	Strain No	Diameter (Mm)	Laccase U/Ml	Lignin Peroxidase U/Ml	Manganese Peroxidase U/Ml
1	*KU1001*	23	350	412	200
2	*KU1002*	3	45	54	40
3	*KU1003*	16	244	302	--
4	*KU1004*	17	255	206	35
5	*KU1005*	--	--	--	--
6	*KU1006*	3	47	54	--
7	*KU1007*	--	--	--	--
8	*KU1008*	--	--	--	--
9	*KU1009*	32	486	576	60
10	*KU1010*	26	390	468	--
11	*KU1011*	24	360	432	25
12	*KU1012*	6	90	108	--
13	*KU1013*	13	195	234	--
14	*KU1014*	12	180	216	--
15	*KU1015*	16	240	188	--
16	*KU1016*	18	270	288	26
17	*KU1017*	12	180	216	--
18	*KU1018*	11	162	191	--
19	*KU1019*	07	105	126	--
20	*KU1020*	23	345	412	--
21	*KU1021*	3	47	54	--
22	*KU1022*	--	--	--	--
23	*KU1023*	--	--	--	--

Evolutionary relationships of 11 taxa (Fig. 3) **:** The evolutionary history was inferred using the Neighbor-Joining method [25]. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed [26]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method [27] and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 573 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

DISCUSSION

In this study, we evaluated the enzyme producing potential of white rot fungi and still several approaches have to explore in order to increase

Accesion No	Description	Max score	Total score	Query coverage	E-value	Max iden
DQ327661.1	*Pycnoporus sp. DIS 343f*	1125	1125	100%	0.0	100%
AY855912.1	*Trametes elegans voucher TENN 054870*	1090	1090	98%	0.0	99%
AY351921.1	*Trametes lactinea strain Chen 550*	1088	1088	98%	0.0	99%
FJ372693.1	*Pycnoporus cf. sanguineus 8R_1_1*	1066	1066	96%	0.0	99%
AF261536.1	*Pycnoporuscinnabarinusstrain DAOM72065*	1064	1064	100%	0.0	98%
AY586703.1	*Pycnoporus cinnabarinus*	1057	1057	100%	0.0	98%
AJ488128.1	*Pycnoporus cinnabarinus,strain CulTENN10140 SBI 2*	1048	1048	98.00%	0.0	98%
AY684160.1	*Pycnoporus sp. ZW02.30 isolate AFTOL-ID 772*	1044	1044	100%	0.0	97%
AF518625.1	*Junghuhnia subundata strain LR-38938*	1040	1040	100%	0.0	97%
AF393074.1	*Pycnoporus cinnabarinus*	1038	1038	99.00%	0.0	97%

Table: 2 Blast report showing the accession no, description, max score, total score query coverage max identity, Evalue of DQ327661.1, AY855912.1, AY351921, FJ372693.1, AF261536.1, AY586703.1, J488128, AY684160.1, AF518625.1, AF393074.1

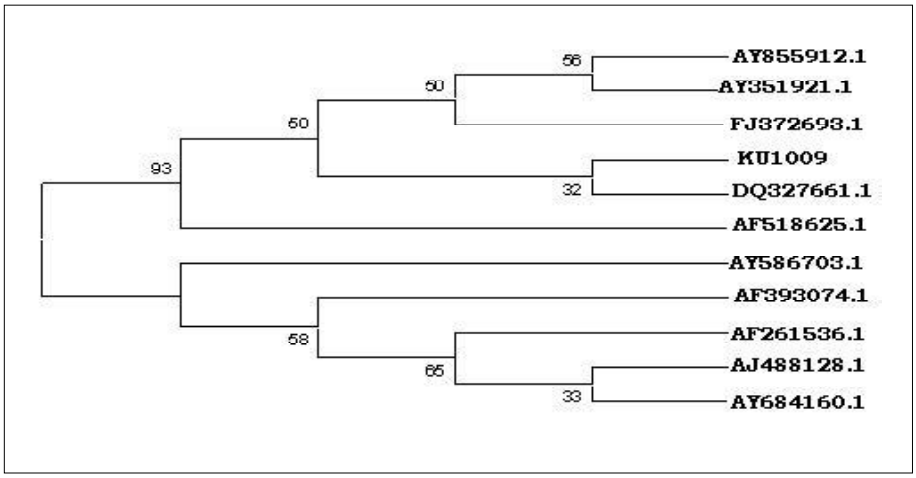

Fig. 3: Evolutionary relationships of 11 taxa

production of lignolytic enzymes. Among these, the most effective appear to be addition to the nutrition medium of specific inducers (or stimulators) of syntheses of these enzymes.

The internal transcribed spacer (ITS) regions of fungal rDNA have been successfully used for species identification. The fungal strain isolated during this research were described as *Pycnoporus sp. DIS 343f* based on the genetic (ITS region) characterization. The ITS-1/4 primers, which lie on either side of the ITS1, 5.8S rDNA and cover regions of both the nuclear 18S rDNA and the nuclear 28S rDNA, were the primers of choice because they are considered to amplify DNA sequences from a wide range of fungi [28]. The ITS region is present at a very high copy number in the genome of fungi, as part of tandemly repeated nuclear rDNA which coupled with PCR amplification produces a highly sensitive assay [29]. Earlier, wood decaying fungi were identified by hybridization of immobilized sequence specific oligonucleotide probes with PCR-amplified fungal rDNA ITS [30]. By sequencing the ITS and large subunit rDNA.

The challenges involved in the large-scale production of fungal peroxidases for application in the environmental field are well identified and encouraging advances have been made regarding major issues, such as stability in the presence of peroxide and heterologous overproduction. However, research efforts should still be directed, mainly using DNA recombinant techniques, to solve the remaining challenges. The design and development of an environmental biocatalyst should then focus on the production of an enzyme stable in the presence of hydrogen peroxide and operational conditions, with a high redox potential to be able to oxidize a wide range of xenobiotics or pollutants, and over expressed at large scale in industrial microorganisms. The feasibility of attaining such developments is significant and within reach.

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