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PRODUCTION OF ETHANOL BY CONSORTIA OF BIOMASS DEGRADING ENZYME FROM WOOD ROT FUNGI IN WESTERN GHATS OF KARNATAKA

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ABSTRACT
Fungi are one of the major degraders in the biosphere that help in degrading most of the plant origin polymers like cellulose, hemicellulose and lignin. Lignin is the second most abundant aromatic compound found in plant cell that holds up cellulose and hemicellulose. There is a lot of emphasis that is being put on the fungal degradation of lignin using wood and other lignocellulosic as a renewable source in the production of chemicals, paper products, feeds and fuels and the use of fungi as one of the most potent sources of degrading organisms. In the present study, screening for lignin degrading enzymes were done with 132 isolates and the maximum enzyme producing strains of 10 wood rot fungi samples were taken for molecular characterization using RAPD molecular markers.Isolation of genomic DNA of the 10 wood rot fungi samples was done using phenol-chloroform method and quantified on agarose gel. The obtained genomic DNA was further subjected tocharacterization using RAPD-PCR method with 06 random primers OPA2, OPA5, OPA7,OPA8, OPD3 and OPC2. The amplified PCR products were analyzed by Agarose Gel Electrophoresis and was observed under UV Transilluminator. Analysis of the base pairs of the bands was done by Bio-Rad Gel Doc system. A total of 172 fragments were generated in the 10 isolates with 6 primers. Dendrogram analysis of the gels were done which gave a close relation of each DNA samples. The results indicate that some strains were genetically more similar and few diverse. The current study shows samples 30, 40, 41, 113 and 124 are genetically closely related.

Keywords: Wood rot fungi, PCR, Ligninase enzyme, RAPD, Molecular markers.

INTRODUCTION
Biomass get decayed, rotten or decomposed every year and fungi are one of the most common organisms that are responsible for decay of wood (Seweta Srivastava *et al.,* 2013). The fungi that grow on wood are called as lignicolousfungi that includes Ascomycetes and Basidiomycetes under them (Seweta Srivastava *et al.,* 2013). Wood rot fungi are one of the major fungal degraders in the biosphere that degrade most of the plant origin polymers like cellulose, hemicellulose and lignin (T.M. D'Souza and Reddy, C.A. 1994). Of these, the brown rot fungi decompose wood components like cellulose and hemicellulose efficiently but has the limitation to degrade lignin while on the other hand, white rot fungi mineralize these plant polymers to CO_2 completely (Kirk. T.K. and R.L. Farrell, 1987).Depending on the wood residue left after degradation, wood decay is classified into two different types such as brown rots, white rots, and soft rots (Seweta Srivastava *et al.,* 2013). Certain basidiomycetes commonly known as white rot fungi completely degrade the woody plant cell wall material (Eriksson*et al.,* 1990).These fungi have the capacity to degrade, depolymerize and mineralize all the cell wall polymers like cellulose, hemicellulose and the recalcitrant polymer like lignin which are all commonly associated with forest litter and woody debris (Amber Vanden Wymelenberg*et al.,* 2011). These types of degradation generally requirevery complex hydrolytic and extracellular oxidative enzyme systems that have been extensively studied in the model white rot fungi like *Phanerochaete chrysosporium* (Kirk. T.K. and R.L. Farrell, 1987). White rot causing basidiomycetes fungi are very efficient in degrading lignin naturally (Eriksson*et al.,* 1990).

Molecular techniques are becoming very important for the taxonomic and phylogenetic relationship studies among different fungi (P.J. Zambino, 1993). By the use of DNA based molecular techniques like Random Amplified Polymorphic DNA Polymorphism (RAPD), Amplified Fragments length

Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP) or DNA sequence analysis (nSSU and mtSSU), limitations of identification of various wood rot fungi or mushroom strains based on a few morphological characters can be overcome.

Any of the molecular methods mentioned above could be combined with morphological methods to make identification of fungal species reliable (R.S. Khush*et al.,* 1992). The random amplified polymorphic DNA (RAPD) is a convenient method in the detection of genetic diversity amongst basidiomycetes (Moore D and Chiu SW, 2001). This method has been particularly successful when applied to check the strains of various wild mushrooms with different origins (Moore D and Chiu SW,2001). RAPD have been used to examine material from the genera *Agaricus*, *Coprinus* and *Lentinula* (A.J. Moore*et al.,* 2001).Genetic Diversity Characterization of *Pleurotus* strains by Random Amplified Polymorphic DNA Fingerprinting has been performed by various workers (M.K. Yadav*et al.,* 2017). Fruiting body observations provide information about the fungi on the surface. In addition, evolutionary relationships cannot be determined accurately through the study of morphology alone (H.B.A. Ben, K. Garrett 2016)**.**

In this report, we have concentrated on the molecular characterization of 10 different wood rot fungi samples responsible for ligninase enzyme production using RAPD molecular markers.

MATERIALS AND METHODS

Isolation of Genomic DNA

Genomic DNA was isolated by phenol-chloroform method. The wood rot fungi strains were grown in 60ml sterile Potato Dextrose Broth for 10-14 days at ambient temperature on shaker (150 rpm). Mycelial mess was filtered through Whatman filter paper, blotted dry, taken in porcelain, frozen in -20^{o}C overnight and crushed to fine powder with the help of porcelain pestle. About 200mg of finely powdered mycelia was added to 500µl Extraction buffer (100mM Tris HCl, 50mM EDTA, 500mM NaCl, 10mM 2-Mercaptoethanol pH 8.0) and was mixed well by vigorous vortexing. 70 µl of 20% SDS was added along with 3 µl of Proteinase K and mixed thoroughly by vortexing and was incubated at 65^{o}C for 10 minutes on a heating block. 170 µl of 5M Potassium acetate was added and mixed thoroughly by vertexing and the tubes were incubated on ice for 20 minutes. 500 µl of chloroform was added to the same tube and mixed thoroughly and centrifuged at 11000 rpm for 10 minutes at 4^{o}C. supernatant was transferred to a new tube and 500 µl of ice cold iso propanol was added slowly from the side of the tube and the contents were mixed by gently inverting the tubes and then centrifuged at 10000 rpm for 10 minutes at 4^{o}C. supernatant was discarded and the pellets obtained were washed twice with ice cold 70% ethanol and centrifuged at 10000 rpm for 5 minutes. After air drying the pellets with no trace of alcohol, the pellets were dissolved in Tris EDTA buffer pH 8.0(10mM Tris, 1mM EDTA). The obtained DNA was further treated with RNAse enzyme to remove RNA contamination. The quality of isolated genomic DNA was determined by Agarose Gel electrophoresis.

RAPD-PCR Amplification

Random Amplification of Polymorphic DNA using PCR method was performed for isolated 10 genomic DNA samples with 06 random primers such as OPA2 (TGCCGAGCTG),OPC2(GTGAGGCGTC), OPD3(GTCGCCGTCA),OPA5(AGGGGTCTTG), OPA7(GAAACGGGTG) and OPA8(GTGACGTAGG).PCR was set up for 25 µl of reaction mixture volume as, 10X buffer(2.5 µl), dNTPs (1.5 µl), primer (1 µl), DNA (2 µl), Taq polymerase (2 µl), Nuclease Free Water (16 µl) for each sample. The thermal cycle profiles for 45 cycles were as follows, initial denaturation at 94^{o}C for 5 minutes, denaturation at 94^{o}C for 30 seconds, annealing at 34^{o}C for 30 seconds, extension at 72^{o}C for 1 minute and final extension at 72^{o}C for 5 minutes for primers OPA2, OPC2 and OPD3. On the other hand, the thermal cycler profiles for 45 cycles were as follows, initial denaturation at 94^{o}C for 5 minutes, denaturation at 94^{o}C for 40 seconds, annealing at 32^{o}C for 40 seconds, extension at 72^{o}C for 1 minute 20 seconds and final extension at 72^{o}C for 5 minutes for primers OPA5, OPA7, OPA8.

Visualization of RAPD-PCR products by Agarose Gel Electrophoresis

PCR products of all 11 primers were separated by 1% Agarose gel, stained with Ethidium bromide using 1X Tris Acetate EDTA buffer. 10 µl of sample(6 µl of amplified PCR product and 4 µl of tracking dye) was loaded into each well with 100bp DNA ladder. Electrophoresis was carried out at 80V and was observed under UV Transilluminator.

RESULTS

Isolation of Genomic DNA

Genomic DNA of 10 wood rot fungi samples(numbered as 30,40,41,61,75,86,104,113,123,124) were isolated using phenol-chloroform method and were checked for the quality by Agarose Gel Electrophoresis and observed under UV Transillumination

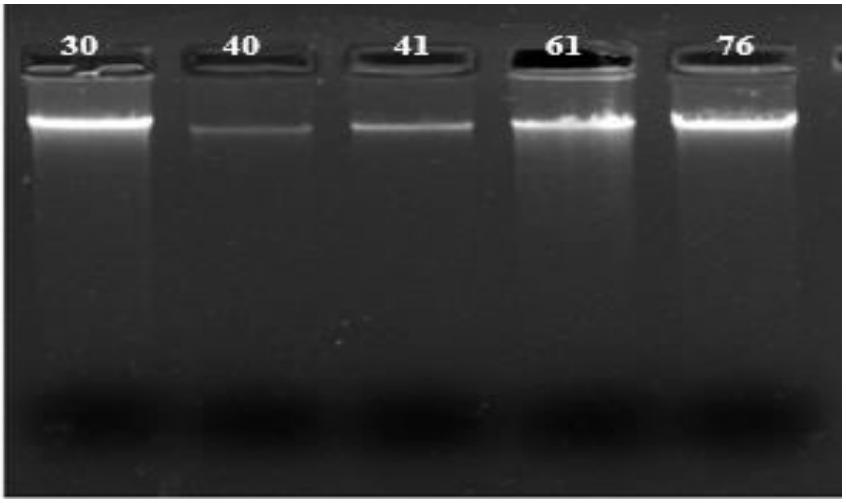


Fig.1: Genomic DNA isolated from wood rot fungi samples 30,40,41,61 and 76

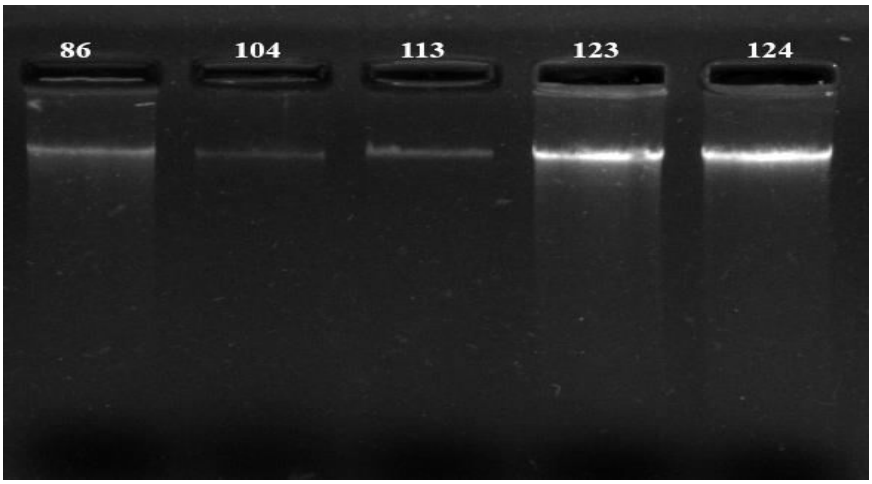


Fig.2: Genomic DNA isolated from wood rot fungi samples 86,104,113,123 and 124

Visualization of RAPD-PCR Products by Agarose Gel Electrophoresis

Isolated genomic DNA of 10 wood rot fungi were subjected to PCR using RAPD technique with 06 random primers such as OPA2 (TGCCGAGCTG), OPC2 (GTGAGGCGTC), OPD3 (GTCGCCGTCA), OPA5 (AGGGGTCTTG), OPA7 (GAAACGGGTG) and OPA8 (GTGACGTAGG). The PCR products were loaded on Agarose Gel and observed under UV Transillumination.

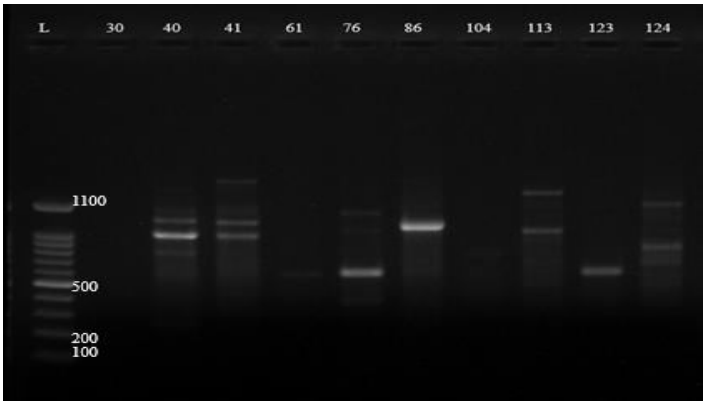
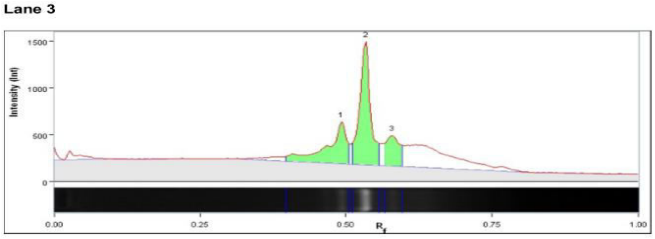


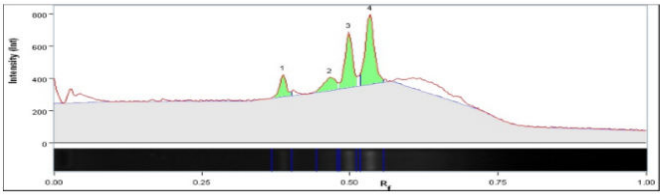
Fig. 3: PCR amplification of 10 wood rot fungi with random primer OPA 2. L represents 100bp DNA Ladder followed by 10 DNA samples.



Band No.	Band Label	Base Pairs (bp)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		1,057.2	0.494	192,647	N/A	N/A	31.8	19.9
2		1,004.0	0.536	314,563	N/A	N/A	51.9	32.5
3		800.0	0.581	98,339	N/A	N/A	16.2	10.2

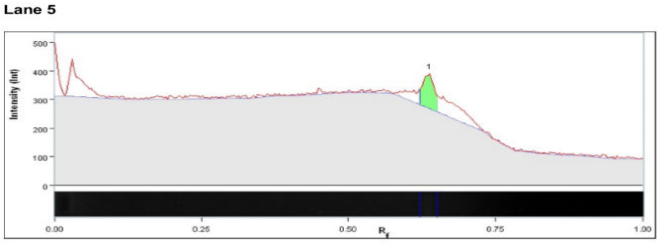
Fig. 4: Lane 3 in Fig. 3 has 3 bands which are 1057.2 bp, 1004 bp and 800 bp respectively

Lane 4



Band No.	Band Label	Base Pairs (bp)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		1,100.0	0.390	20,010	N/A	N/A	11.0	6.4
2		1,091.3	0.468	21,025	N/A	N/A	11.6	6.7
3		1,048.8	0.500	57,130	N/A	N/A	31.5	18.2
4		1,004.0	0.536	83,172	N/A	N/A	45.9	26.5

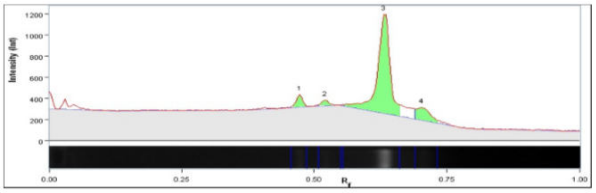
Fig. 5: Lane 4 in Fig. 3 has 4 bands which are 1100 bp, 1091.3 bp, 1048.8 bp and 1004 bp respectively



Band No.	Band Label	Base Pairs (bp)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		578.5	0.640	25,317	N/A	N/A	100.0	19.2

Fig. 6: Lane 5 in Fig. 3 has 1 band which is 578.5 bp.

Lane 6



Band No.	Band Label	Base Pairs (bp)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		1,082.7	0.474	16,559	N/A	N/A	5.3	4.2
2		1,020.1	0.523	9,222	N/A	N/A	3.0	2.3
3		600.0	0.633	249,400	N/A	N/A	80.0	63.6
4		391.2	0.705	36,685	N/A	N/A	11.8	9.3

Fig. 7: Lane 6 in Fig. 3 has 4 bands which are 1082.7 bp, 1020.1 bp, 600 bp and 391.2 bp respectively

Fig.8: Lane 11 in Fig. 39 has 7 bands which are 1100 bp, 1082.7 bp, 1000 bp, 900 bp, 710.5 bp, 634.6 bp and 222.5 bp respectively

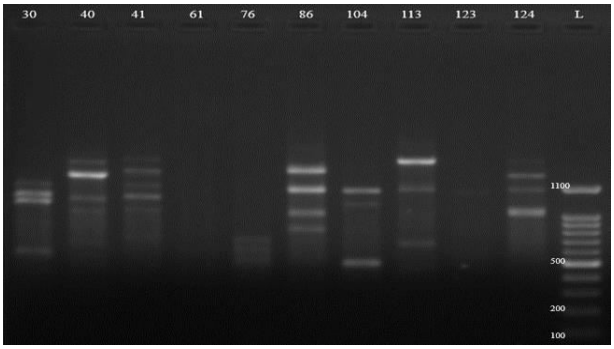
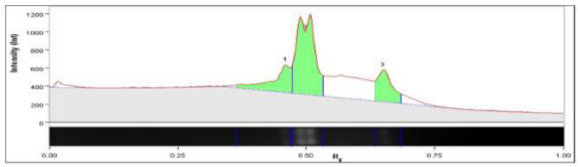


Fig. 9: PCR amplification of 10 wood rot fungi with random primer OPD 3. L represents 100bp DNA Ladder followed by 10 DNA samples.

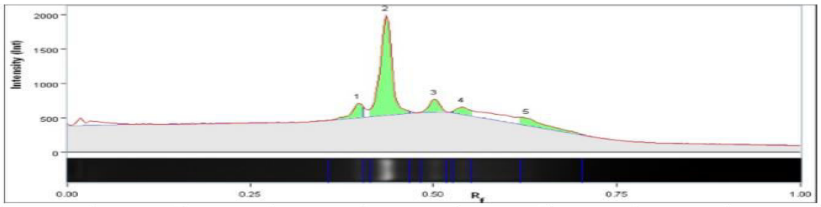
Lane 1



Band No.	Band Label	Base Pairs (bp)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		1,100.0	0.462	133,748	N/A	N/A	21.0	14.3
2		1,075.1	0.500	376,362	N/A	N/A	59.0	40.2
3		620.9	0.651	128,122	N/A	N/A	20.1	13.7

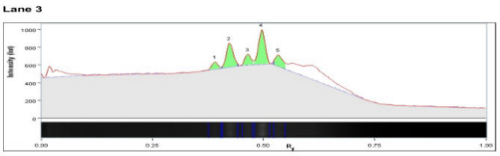
Fig.10: Lane 1 has 3 bands which are 1100 bp, 1075.1 bp and 620.9 bp respectively

Lane 2



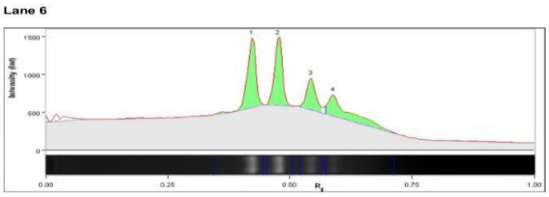
Band No.	Band Label	Base Pairs (bp)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		1,100.0	0.399	41,122	N/A	N/A	9.2	7.2
2		1,100.0	0.437	294,466	N/A	N/A	65.6	51.7
3		1,071.0	0.503	35,177	N/A	N/A	7.8	6.2
4		1,023.1	0.541	24,476	N/A	N/A	5.5	4.3
5		700.0	0.629	53,534	N/A	N/A	11.9	9.4

Fig.11: Lane 2 has 5 bands which are 1100 bp, 1100 bp, 1071 bp, 1023.1 bp and 700 bp respectively



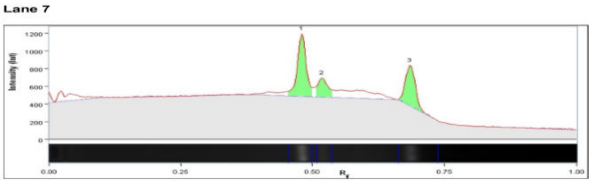
Band No.	Band Label	Base Pairs (bp)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		1,100.0	0.393	12,441	N/A	N/A	7.3	3.5
2		1,100.0	0.425	47,792	N/A	N/A	28.1	13.4
3		1,100.0	0.465	20,126	N/A	N/A	11.8	5.7
4		1,079.2	0.497	64,757	N/A	N/A	38.0	18.2
5		1,031.0	0.535	25,201	N/A	N/A	14.8	7.1

Fig.12: Lane 3 has 5 bands which are 1100 bp, 1100 bp, 1100 bp, 1079.2 bp and 1031 bp respectively



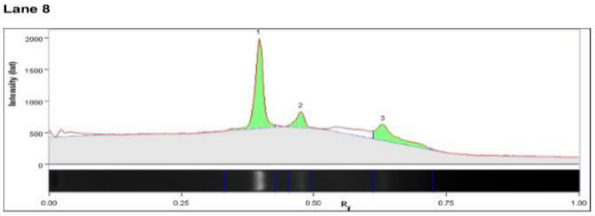
Band No.	Band Label	Base Pairs (bp)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		1,100.0	0.425	229,506	N/A	N/A	31.7	29.3
2		1,100.0	0.478	209,902	N/A	N/A	29.0	26.8
3		1,019.2	0.544	112,462	N/A	N/A	15.5	14.4
4		855.7	0.591	173,130	N/A	N/A	23.9	22.1

Fig.13: Lane 6 has 4 bands which are 1100 bp, 1100 bp, 1019.2 bp and 855.7 bp respectively



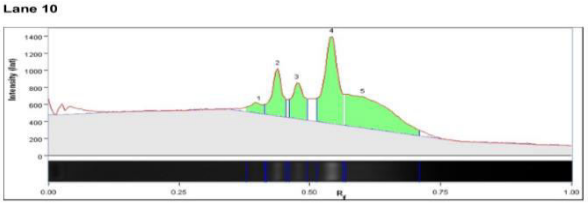
Band No.	Band Label	Base Pairs (bp)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		1,100.0	0.481	146,624	N/A	N/A	48.2	30.7
2		1,050.8	0.519	50,141	N/A	N/A	16.5	10.5
3		516.9	0.686	107,387	N/A	N/A	35.3	22.5

Fig.14: Lane 7 has 3 bands which are 1100 bp, 1050.8 bp and 516.9 bp respectively



Band No.	Band Label	Base Pairs (bp)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		1,100.0	0.399	274,514	N/A	N/A	60.5	46.0
2		1,100.0	0.478	52,432	N/A	N/A	11.6	8.8
3		688.1	0.632	126,614	N/A	N/A	27.9	21.2

Fig.15: Lane 8 has 3 bands which are 1100 bp, 1100 bp and 688.1 bp respectively



Band No.	Band Label	Base Pairs (bp)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		1,100.0	0.406	35,728	N/A	N/A	3.8	3.3
2		1,100.0	0.440	121,887	N/A	N/A	12.9	11.3
3		1,100.0	0.478	112,694	N/A	N/A	11.9	10.4
4		1,019.2	0.544	313,519	N/A	N/A	33.1	28.9
5		800.0	0.604	362,964	N/A	N/A	38.3	33.5

Fig.16: Lane 10 has 5 bands which are 1100 bp, 1100 bp, 1100 bp 1019.2 bp and 800 bp respectively

Dendrogram Analysis:

Dendrogram analysis was carried out using dendrogram analysis tool PyElph 1.4 and the results interpreted as follows,

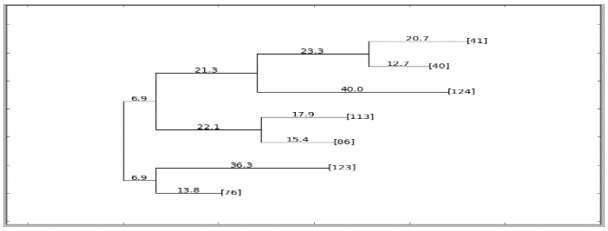


Fig. 17: Dendrogram analysis for DNA samples 40, 41, 76, 86, 113, 123 and 124 with primer OPA 2

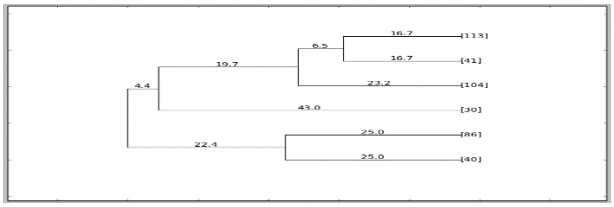


Fig. 18: Dendrogram analysis for DNA samples 30, 40, 41, 86, 104 and113 with primer OPA 5

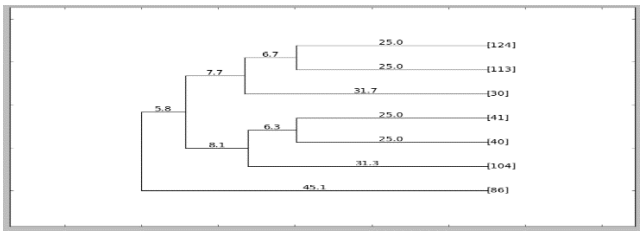


Fig. 19: Dendrogram analysis for DNA samples 30, 40, 41, 86, 104, 113 and 124 with primer OPA 8

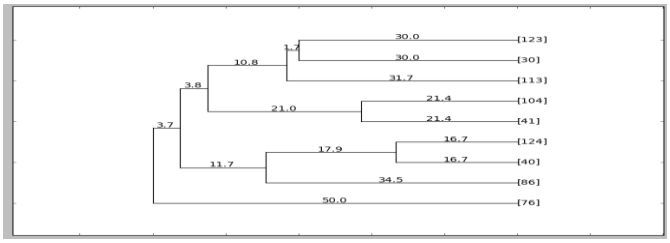


Fig. 20: Dendrogram analysis for DNA samples 30, 40, 41, 76, 86, 104, 113, 123 and 124 with primer OPA 7

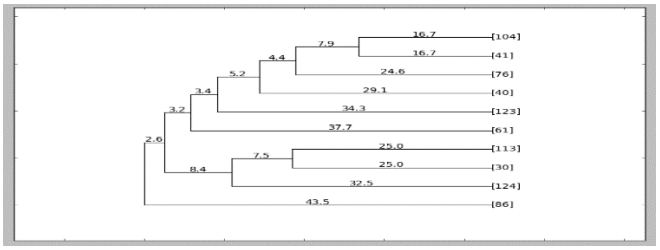


Fig. 21: Dendrogram analysis for DNA samples 30, 40, 41, 61, 76, 86, 104, 113, 123 and 124 with primer OPC 2

Genetic variability among 10 isolates of wood root fungi were carried out using RAPD-PCR technique. All the primers produced clear bands varying in numbers for different samples with primer OPA2 showing amplicons for samples 40, 41, 76, 86, 113, 123 and 124. Primer OPA5 showed amplification for samples 30, 40, 41, 86, 104 and 113, with primer OPA8 showing amplification for samples 30, 40, 41, 86, 104, 113 and 124.Primer OPA7

exhibited amplicons forsamples 30, 40, 41, 76, 86, 104, 113, 123 and 124.Primer OPC2 for samples 30, 40, 41, 61, 76, 86, 104, 113, 123 and 124 and primer OPD3 for samples 30, 40, 41, 86, 104, 113 and 124. A total of172 fragments were generated in 10 isolates with 6 primers.

Pattern of polymorphism	OPA2	OPA5	OPA8	OPA7	OPC2	OPD3
Total No. of bands	19	21	33	25	44	30

Dendrogram representing the genetic relationship among the isolates based on the amplification potential and reproducibility of 06 RAPD primers is represented as follows on performing dendrogram analysis of the gel, samples 41 and 40 are closely related while 113 and 86 are closely related when subjected to primer OPA2,samples 113 and 41 are closely related with primer OPA5,samples 124 and 113 are closely related while samples 41 and 40 are closely related to each other with primer OPA8, samples 124 and 40 are closely related &samples 113 and 30 are closely related while samples 104 and 41 are closely related with primer OPA7, samples 104 and 41 & 113 and 30 are closely related with primer OPC2, samples 124 and 40,while 86 and 41 and 104 and 30 are closely related with primer OPD3. The above results indicate that some strains were genetically more similar and few diverse. The current study shows samples 30, 40, 41, 113 and 124 are genetically similar.

DISCUSSION

The present study was aimed at determine the genetic diversity using RAPD technique amongst the wood rot fungi isolated from the westernGhats of Karnataka to study its Ligninase activity. RAPD-PCRtechnique is a useful tool for differentiating between species and complementary to methods based upon morphological and pathological characteristics. The genomic DNA was isolated and then analyzed further by RAPD-PCR method, wherein the 10 DNA samples were subjected to 6 random primers OPA2, OPC2, OPD3, OPA5, OPA7 and OPA8. The amplified DNA products wereobserved and analysis of base pairs of the bands was done by Bio-Rad Gel Doc system software. Dendrogram analysis of the gels was also done which gave a close relation of each DNA sample in order to assess the phylogenetic relationship among them. In the present study, and data given by RAPD analysis, comparison of two different or same members were done.

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