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TO STUDY ABOUT THE MICROBIAL BIOTRANSFORMATION OF LIGNIN FOR VALUE ADDED PRODUCTS

Kanduri Venkata Sadasiva Rao, Research Scholar, Dept. of Biotechnology, Sikkim Professional University
Dr Aakansha Goswami, Professor, Dept. of Biotechnology, Sikkim Professional University

ABSTRACT

Lignocellulosics are the most significant materials for a sustainable human society because they not only form long-term sustainable carbon flow in the ecosystem, but they also fulfill the three major keywords of "Energy, '"Materials," and "Environment". Petroleum (30%), natural gas (23%), coal (22%), renewable (19%), and nuclear (6%) were the primary energy sources in the previous century. 4 percent of crude oil and 31% of natural gas are used in the chemical industry to make platform chemicals and composite materials. Lignocellulosic biomass, such as agriculture and forestry wastes or energy crops, is the world's most abundant and inexpensive renewable resource, serving as a raw material for the manufacturing of biofuels and chemicals and providing around 14% of the world's energy. Not only does biological conversion of plant lignocellulose play a significant role in carbon cycling in terrestrial ecosystems, but it also plays a role in the creation of second-generation biofuels and biochemicals. Lignocellulosic biomass may be transformed into a variety of high-value products, including value-added fine chemicals.

KEY WORDS: Lignocellulose, biomass, carbohydrate polymers, cellulose and hemicellulose.

INTRODUCTION

Lignocellulosic biomass is primarily made up of three components: two carbohydrate polymers (cellulose and hemicellulose), and one non-carbohydrate phenolic polymer (lignin). Cellulose is the major structural polysaccharide produced by polymerization of the disaccharide cellobiose unit, accounting for 30- 50% of lignocellulose's dry weight. It is made up of glucose units that are held together by intramolecular and intermolecular

hydrogen bonds. Hemicellulose is the second polysaccharide component, accounting for 15-30% of the dry weight of the plant cell wall. Hemicelluloses are found embedded in the cell walls of plants, and one of their primary roles is to bind cellulose microfibrils to reinforce the cell wall. Xylan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan are among the heteropolymers that make up hemicellulose, which has a random and amorphous structure. Different 5- and 6-carbon monosaccharide units make up hemicellulose heteropolymers: pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and acetylated sugars. Lignin is the third most important component of lignocellulose, accounting for 15–30% of the dry mass. After cellulose, lignin is the second most abundant carbon source. Lignin acts as a "cellular glue," binding structural carbohydrate moieties together and providing strength, rigidity, and protection against microbial attack to more easily degradable cellulose and hemicellulose polymers (Abdel-Hamid et al., 2013; Isikgor et al., 2015; Mäkelä et al., 2015; Mäkelä et al., 2015).

Lignocellulosic biomass and its components

It is estimated that around 200 1012 kg of terrestrial biomass is created annually over the planet (Hatakka et al., 2011). Lignocellulosic biomass is the only ecological and renewable primary energy resource that is used to produce a variety of value-added goods in addition to providing alternative energy sources (Pérez et al., 2002). Only a small portion of the cellulose, hemicellulose, and lignin produced as by-products in agriculture or forestry is naturally recycled (Pérez et al., 2002; Sánchez, 2009). The three primary components of lignocellulosic material are cellulose (30-50 percent), hemicellulose (15-35 percent), and lignin (10-20 percent). Cellulose and hemicelluloses account for over 70% of total biomass and are densely interconnected with lignin via covalent and non-covalent interactions, resulting in a structure that is highly durable and resistant to treatment (Sánchez, 2009; Limayem et al., 2012). The conversion of lignocellulosic biomass into value-added goods has recently gotten a lot of press. This area of biomass utilization is now thought to be a viable strategy to reduce negative environmental impact. Furthermore, it is expected that renewable raw resources will be able to replace finite mineral-oil-based raw materials by 2050. (Murzin et al., 2012). The maize ethanol industry and the pulp and paper industry are two industries with well-developed biomass conversion infrastructure. These businesses give opportunities for creating monomeric sugars from lignocellulosic biomass, ethanol production through fermentation, and the conversion of lignin-rich

fermentation wastes and low-quality biomass residues into valuable fuels. These businesses also provide a pathway for biomass or lignin-rich spent pulping liquors (a waste byproduct of the paper and pulp industries) to be converted into fuels, chemicals, and biopower (Kelley, 2007). Because lignin is an economically underutilized, substantial byproduct of these sectors, where it is generally used as a low-cost combustion fuel, novel techniques that can generate value-added products from lignin are required. Aromatic lignin is the only large-scale renewable feedstock made up of aromatics.

RESEARCH METHODOLOGY

Lignocellulosic material for the extraction of lignin

Source of lignocellulosic material for lignin extraction

Wood from Acacia nilotica was obtained locally from a saw mill in Bilaspur, Chhattisgarh, India. Mechanical grinding and sieving were used to create wood dust with a mesh size of 18 mesh.

Organosolv lignin extraction (by different solvents)

The organosolv lignin fractions were extracted utilizing a Dionex Accelerated Solvent Extractor and an accelerated pressurized solvent extraction (PSE) equipment (ASE 150, Thermo Scientific, India). PSE was used to extract various lignin fractions from A. nilotica wood using acetone (AC), chloroform (CH), ethanol (ET), ethyl acetate (EA1), methanol (ME), and water (AQ1). Temperature was 60°C, static time was 7.5 minutes, rinse volume was 60%, nitrogen purge time was 300 seconds, static cycle 2 was used, pressure was 1700 psi, and the solvent volume was 150 mL. To remove any solid particles, the samples were centrifuged for 10 minutes at 8000 rpm. The supernatant was collected, vacuum dried, and kept at -20 degrees Celsius.

Isolation of ligninolytic microorganism

Soil samples were taken from the Guru Ghasidas Vishwavidyalaya campus in Bilaspur, C.G., and the effluent discharge site of a paper mill in Amalai, M.P., India, for the isolation of ligninolytic fungus. Soil samples were kept at 4°C in sterile polythene bags until they were isolated. By using the usual dilution approach, bacteria and fungus were isolated in NAM (nutrient agar medium) and PDA (potato dextrose agar) medium, respectively. Ten bacterial strains and 32 obtained pure fungal strains were exposed to Bavendam's test to search for powerful ligninolytic microorganisms (Bavendam, 1928; Tanabe et al., 1989; Shleev et al., 2004). On the basis of color removal capacity in lignin-containing basal media, the selected fungal strains from the Bavendam test were further screened for their

ability to breakdown lignin. These two screening processes resulted in the selection of two powerful fungi, F10 and APF4, for further lignin degradation research. Selected fungi were grown on a slant of malt extract agar (MEA) containing 5% (w/v) malt extract and stored at 4 oC for further research.

RESULTS AND DISCUSSION

Determination of the wood components content

The TAAPI small scale approach was used to undertake a quantitative compositional study of Acacia nilotica wood (Table 4.1). The A. nilotica wood utilized in the study was found to contain a high amount of extractable lignin (25%) as well as other extractives (7%). The cellulose and hemicellulose content of A. nilotica wood was also high.

Table 1: Quantitative determination of different content of A. nilotica wood.

Component	**Cellulose**	**Hemicellulose**	**Lignin**	**Extractives**	
% w/w	39.53 ± 3.87	29.23 ± 2.12	25.85 ± 3.45	7.32	3.24

Extraction of lignin

Organosolv lignin extraction (by different solvents)

Under high pressure and temperature, organosolv lignin fractions were extracted using the pressurized solvent extraction method (PSE). Table 4.2 shows the percent yield of the organosolv lignin.

Table 2: Percent yield, TPC and TCC of the organosolv lignin by PSE method.

Sample	**% Yield**	**TPC**	**TCC**
Acetone (AC)	7.22 ± 0.87***	358.57 ± 17.01***	1.45 ± 0.07***
Aqueous (AQ1)	2.08 ± 0.12	538.80 ± 29.44***	3.32 ± 0.11***
Chloroform (CH)	4.25 ± 0.45***	260.55 ± 23.24	1.03 ± 0.13*
Ethyl acetate (EA1)	2.30 ± 0.20*	513.40 ± 43.15***	0.95 ± 0.07
Ethanol (ET)	2.10± 0.32NS	591.03 ± 27.97***	3.00 ± 0.08***
Methanol (ME)	5.07 ± 0.60***	358.67 ± 21.15***	3.54 ± 0.10***

Values represent mean ± standard deviation (SD) of three independent experiments (n=3). Values within in a column are significantly different according to one way ANOVA, NS-Non significant, ***p <0.001, **p <0.01 and *p <0.05. AQ1, CH and EA1 was taken as reference for one way ANOVA analysis in % yield, TPC and TCC.

Acetone (AC) extraction yielded a high percent yield, while aqueous (water) extraction had a low percent yield. AC>ME>CH>EA1>ET>AQ1 was the percent yield order.

Lignin extraction with alkali (soda), acidic, and hot water

Table 4.3 compares the percent yield of lignin extracted using alkali (0.1-0.5 N), acidic, and hot water methods. AL4 (extracted with 0.4 N NaOH) yielded the highest percent yield in the alkali process, while AL1 yielded the lowest (extracted by 0.1 N NaOH). AL4>AL5>AL3>AL2>AL1 was the order of the percent yield in the alkali procedure. In the acidic extraction procedure, sulfuric acid extract had a higher percent yield of lignin than acetic acid extract. With a yield of 3.54 percent, the hot water extraction process has the lowest yield.

Table 3: Percent yield, TPC and TCC of the alkali, acidic and hot water lignin.

Lignin Sample	**Yield %**	**TPC**	**TCC**
AL1	13.83 ± 1.50***	232.47 ± 6.4***	1.94 ± 0.13**
AL2	17.15 ± 0.84***	174.00 ± 3.0**	1.42 ± 0.15*
AL3	25.70 ± 0.45***	202.28 ± 3.7***	1.01 ± 0.02
AL4	27.45± 0.57***	187.75 ± 1.9***	2.93 ± 0.16**
AL5	27.17 ± 0.63***	105.38 ± 5.7^{NS}	4.75 ± 0.24***
Acidic (by H_2SO_4) ACL1	14.56± 0.66***	98.57 ± 4.5	9.22 ± 0.26***
Acidic (by Acetic acid) ACL2	13.35 ± 0.58***	110.42 ± 3.5*	7.07 ± 0.25***
HW	2.83 ± 0.09	295.21 ± 7.2***	6.08 ± 0.28***

Values represent mean ± standard deviation (SD) of three independent experiments (n=3). Values within in a column are significantly different according to one way ANOVA, NS-Non significant, ***p <0.001, **p <0.01 and *p <0.05. HW, ACL1 and AL3 was taken as reference for one way ANOVA analysis in % yield, TPC and TCC.

Fractions of alkali lignin (0.3 N NaOH) extracted by Successive SolventExtraction (SSE)

Successive Solvent Extraction (SSE) was used to fractionate alkali lignin. 250 mL of alkali lignin solution (extracted by 0.3 N) of A. nilotica wood was fractionated using n-hexane (HX), diethyl ether (DE), ethyl acetate (EA2), and n-butanol (BU). Table 4.4 displays the yield of each fraction.

Table 4: Yield and TPC of the lignin fractions extracted by SSE.

Lignin Sample	Yield	TPC
Butanol (BU)	72.1 mg	355.36 ± 10.22***
Diethyl ether (DE)	32.4 mg	303.03 ± 18.18***
Ethyl acetate (EA2)	29.0 mg	285.11 ± 27.42***
Aqueous (AQ2)	15.29 g	144.42 ± 32.34**
Hexane (HX)	38.5 mg	39.10 ± 9.792

Values represent mean ± standard deviation (SD) of three independent experiments (n=3). Valueswithin in a column are significantly different according to one way ANOVA, NS- Non significant, ***p <0.001, **p <0.01 and *p <0.05. HX was taken as reference for one way ANOVA analysisfor TPC.

Biodegradation study of alkali lignin by selected ligninolyticfungus strain (F10 and APF4)

Screening and isolation of ligninolytic microorganism

32 morphologically distinct fungal strains were recovered on PDA and 10 bacterial strains were identified on NAM during the screening of ligninolytic microorganism from different soil samples. 18 fungal strains were chosen from among these microorganisms based on their capacity to breakdown polyphenol in Bavendam medium. The ligninolytic fungus produces phenol oxidase (a combination of ligninolytic enzymes such as lignin peroxidase, MnP, and laccase), which produces a black color zone (positive test) around the mycelium. On Bavendam media, none of the bacterial strains tested positive.

The color removal capacity of the eighteen fungus was then tested in lignin-containing basal media. Following that, two powerful lignin degrading fungi, F10 and APF4, were chosen for lignin degradation investigations.

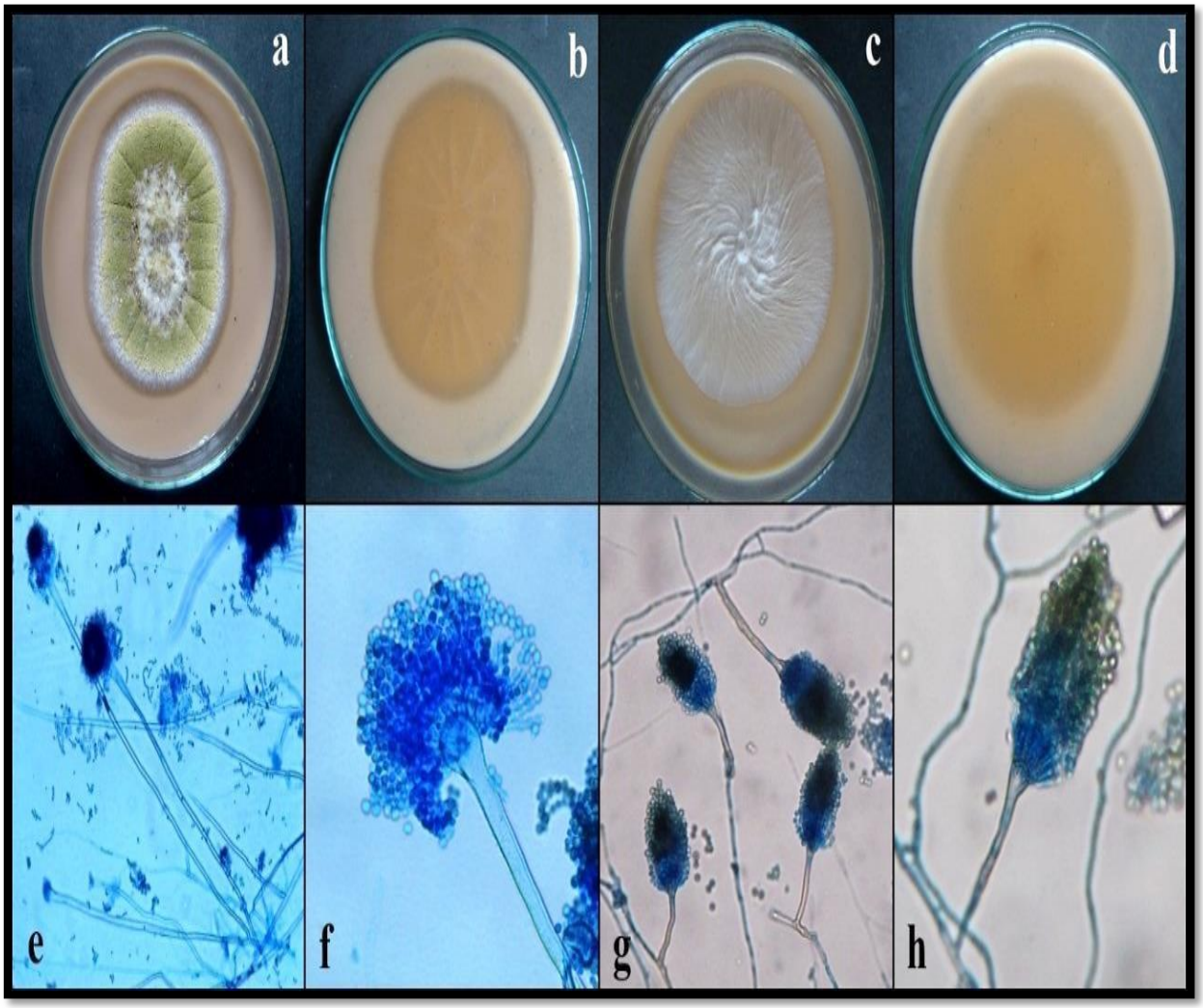

Fig. 1: Bavendam test of selected fungus F10 (a- front; b- back view of culture plate) **and APF4** (c- front; d- back view of culture plate) **and microscopic view of F10**(e-conidiophore and conidial heads; f -conidia) **and APF4** (g-conidiophore and conidialheads and h- conidia).

Identification of fungus strain

A polyphasic technique was used to identify the selected strains (based on their microscopic, morphology and 18S rRNA sequencing). Both fungi were inoculated in Czapak-Dox agar (CDA), cultured for five days, and then microscopically examined.

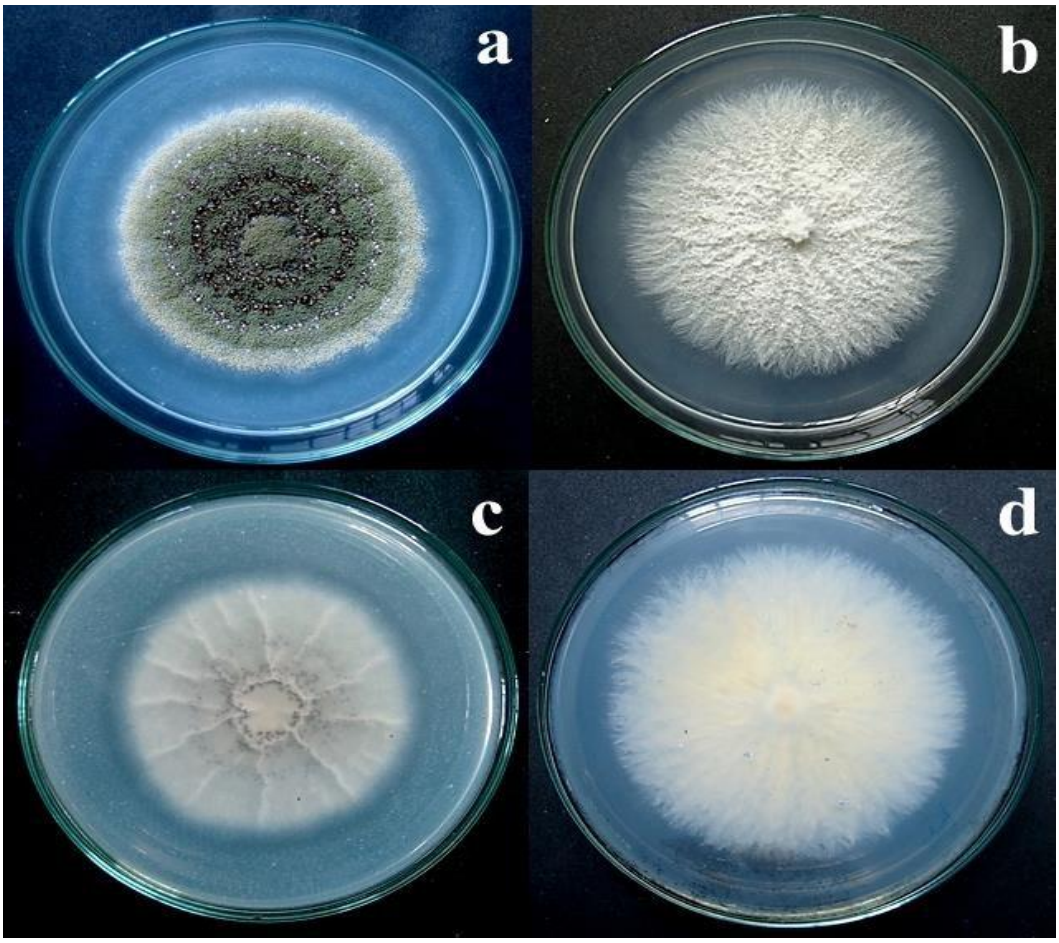

Fig. -2: Five day old culture of selected fungus A. flavus (F10) and E. nidulans *(APF4) on Czepak-Dox Agar.*

(a) A. flavus (F10) front view; (b) E. nidulans (APF4) front view;
(c) A. flavus (F10) back view; (d) E. nidulans (APF4) back view

F10 colonies on CDA medium grow at a moderate rate, with a powdery green texture and a diameter of 3-4.5 cm, whereas APF4 colonies grow at a slower rate, with a cottony appearance and a diameter of 3-4 cm. F10 has a mycelium consisted of hyaline, branching, aseptate, smooth-walled hyphae with a width of 3-12 m, according to microscopic examinations. Conidiophores are smooth-walled and spherical, with a length of 200-500 m and a width of 8-12 m; conidial heads are loosely radiating and have a width of 30-60 m; metulaes and phialides cover 60 percent to practically the whole vesicle.

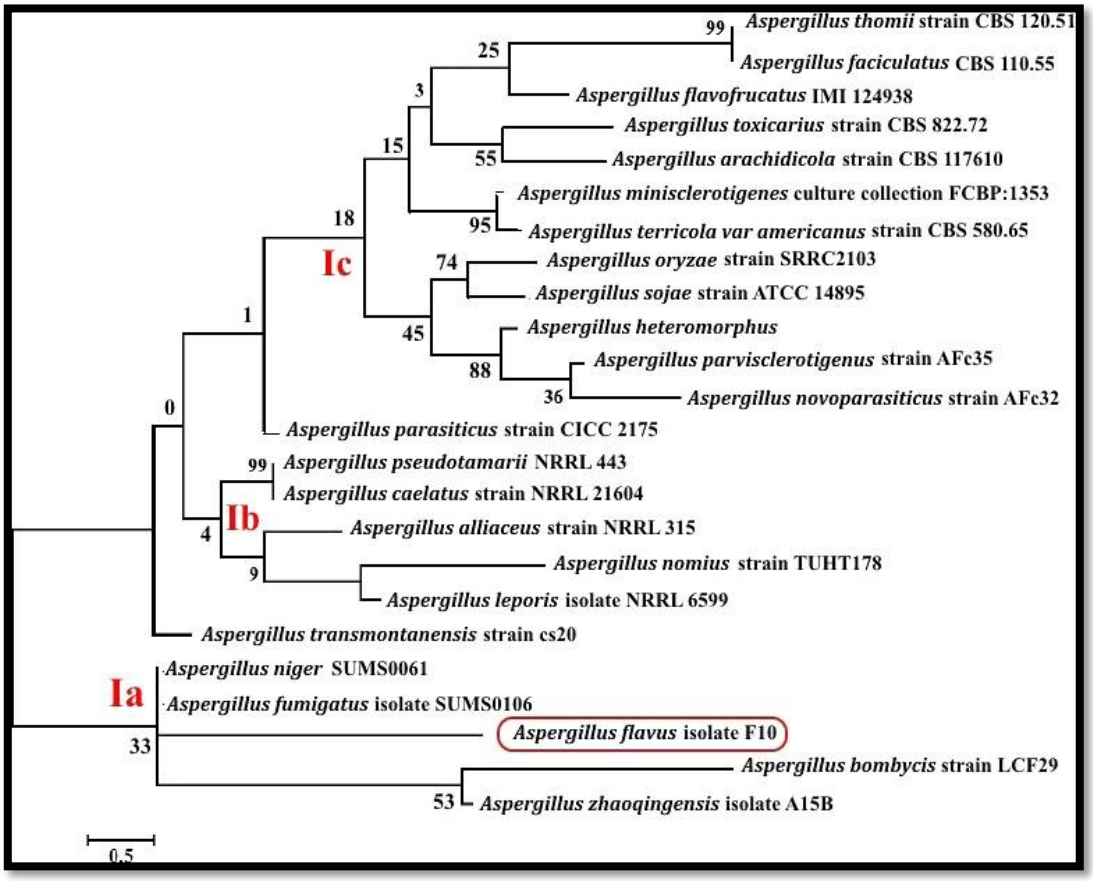

Fig. 3: Phylogenetic tree (dendrogram) of A. flavus (F10) based on the result of 18S rRNA gene sequence comparison by Maximum Likelihood (ML) method.

Conidiophores of the APF4 strain are hyaline, simple, thick-walled, inflated globosely or ellipsoidally at the apex (called vesicles), length 120-250 m, width 8-12 m; bearing spore heads composed of catenulate conidia borne on uniseriate phialides on vesicles: conidial heads, dark in color, compactly columnar with coni. The isolated fungi F10 and APF4 have significant homology similarity (>98%) with Aspergillus flavus and Emericella nidulans, according to the results of partial 18S rRNA gene sequence alignment based on BLAST analysis. The sequences have been allocated accession codes KC911631.1 (F10) and KC911632.1 (APF4) in the NCBI Genomic Bank nucleotide database (Appendix - II). In Figures 3 and 4, a phylogenetic tree dendrogram was created. The evolutionary history was deduced using the Kimura 2-parameter model and the

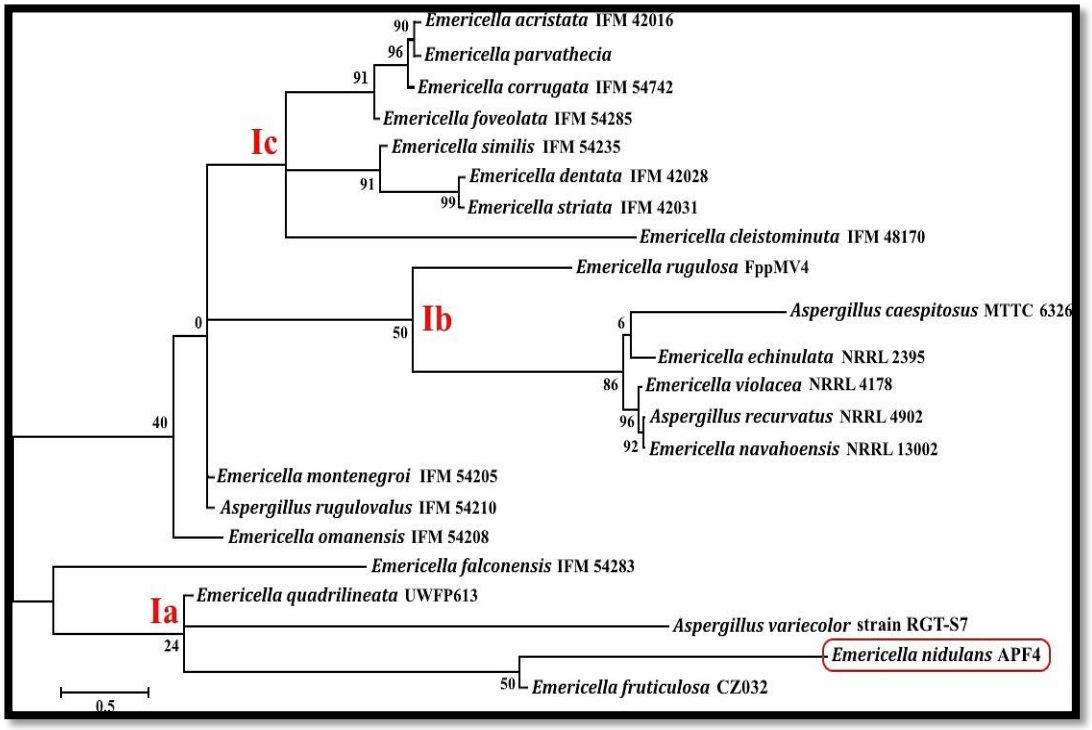

Fig. 4: Phylogenetic tree (dendrogram) of E. nidulans (APF4) based on the result of 18S rRNA gene sequence comparison by Maximum Likelihood (ML) method.

Maximum Likelihood technique. Figures 3 and 4 show the trees with the highest log probability (-11782.0052 and -10605.2885 for A. flavus (F10) and E. nidulans (APF4), respectively. Next to the branches is the proportion of trees in which the related taxa clustered together. The initial tree(s) for the heuristic search were automatically generated by applying the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances calculated using the Maximum Composite Likelihood (MCL) technique, and then picking the topology with the best log likelihood value. The branch lengths are measured in the number of substitutions per site, and the tree is depicted to scale. For A. flavus (F10) and E. nidulans (APF4), the study required 22 and 24 nucleotide sequences, respectively. Gaps and missing data were removed from all positions. The final dataset for A. flavus and E. nidulans had 430 and 509 locations, respectively. MEGA6 was used to conduct evolutionary analysis.

The phylogenetic tree dendrogram for A. flavus is shown in Fig. 4.13. (F10). Dendrogram analysis found that 22 of the 24 fungal species studied were organized into three primary groupings (Ia, Ib and Ic). The isolate A. flavus (F10) was placed in the Ia group alongside A. niger, A. fumigatus, A. bombycis, and A. zhaoqingensis, and showed close resemblance to A. niger and A. fumigatus.

Figure 4. shows the phylogenetic tree dendrogram for E. nidulans (APF4), which was created alongside the 21 fungal species. All 22 fungal species were found to be organized into three primary groups on a dendrogram (Ia, Ib and Ic). E. nidulans (APF4) was clustered with E. falconensis, E. quadrilineata, A. variecolor, and E. fruticulosa in Ia cluster and was shown to be quite near to E. fruticulosa.

CONCLUSION

Plant phenolics, polyphenols, and lignin have gained popularity as a result of their therapeutic benefits, which include antioxidant, antibacterial, anti-inflammatory, cardio-protective, cytotoxic, chemo-preventive, and neuro-protective qualities. Polyphenols are a type of health food supplement that is said to have health-promoting and disease-preventive characteristics. Lignin is a natural polymerized product of optically active p-hydroxycinnamyl alcohol monomers and related monolignols (p-coumaryl, coniferyl, and sinapyl) formed by oxidative reactions. It is the second most abundant natural macromolecule (polyphenolic in nature and 10–35 percent of dry wt. of lignocellulosic biomass). Various inter unit linkages in the monomer and monolignols (e.g. -O-4, -5', -', 5-5', and 4-O-5') result in this polymer. The test plant in this study was Acacia nilotica, often known as "Babul," a multifunctional deciduous tree of the Mimosaseae family native to Central India. This plant includes ellagic acid, isoquercitin, leucocyanadin, kaempferol-7-diglucoside, (+)-catechin derivatives, apigenin derivatives, and other bioactive components. Cold, bronchitis, diarrhoea, dysentery, biliousness, bleeding piles, and leucoderma are among the illnesses that A. nilotica leaves, pod, bark, and root have traditionally been used to cure in the Central area of India. Alkali lignin A3 (0.3 N NaOH alkali lignin) was employed in the lignin biodegradation study. The two ligninolytic fungi were discovered in soil samples taken from the Guru Ghasidas Vishwavidyalaya campus in Bilaspur (C.G.) and near the Orient paper mill's effluent discharge location in Amalai (M.P.). Based on their morphology and high homology in 18S rRNA gene sequences, the fungi were identified as Aspergillus flavus strains (F10; KC911631) and Emericella nidulans (APF4; KC911632).

Lignin degradation is a multi-step process mediated by a variety of oxido-reductase enzymes. Laccase (Lac), Manganese Peroxidase (MnP), and Lignin Peroxidase (LiP) are the major enzymes involved in fungal-mediated lignin breakdown.

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