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TO STUDY OF LIGNIN FOR VALUE ADDED PRODUCTS AND ASSESSMENT OF THEIR INDUSTRIAL APPLICATION

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ABSTRACT

The products collected from the LC-MS analysis will also be useful in determining the MG degradation mechanism by A. flavus. The phytotoxicity and micotoxicity tests also guarantee that the breakdown products are not environmentally damaging. To immobilize the A. flavus, several inert materials such as polyurethane foam, vegetable foam, stainless steel gauge, ash, and clay bricks were utilized (F10). The immobilized fungus has a higher breakdown efficiency and takes less time to degrade. lignin fractions isolated from the wood of A. nilotica using Pressurized Solvent Extraction (PSE) and Successive Solvent Extraction (SSE) were investigated for antioxidant, free radical scavenging, and anticancer activity. The PSE fractions (EA1 and ET) had a high polyphenolic content and reducing power, according to the findings (CH fraction). However, SSE fractions had better antioxidant efficacy as measured by DPPH and ABTS radical scavenging assays (BU, DE and EA1). Six lignin fractions (CH>AQ1>AQ2>DE>EA=EA1) had stronger cytotoxic capacity than the test standard medication (Tamoxifen) against the MCF-7 breast cancer cell line, whereas fractions extracted by SSE (DE and EA) have the best inhibitory potential against human prostate cancer cells. These findings show that lignin extracts from A. nilotica wood have a remarkable potential for preventing disease caused by excessive radical generation, as well as being a promising contender as a natural antioxidant and anticancer agent.

Key Words: Lignin, Value Added Products, And Assessment, Industrial Application

INTRODUCTION

Lignin: A potentially useful resource

In the 1830s, a word derived from the Latin "lignum" (wood) was coined. Bente pointed out that lignin is aromatic in nature, and Benedikt and Bamberger discovered that these lignified materials contain methoxyl groups in the 1890s. Peter Klason (1848–1937), who played a key role in the characterisation of lignin and proposed that lignin was made up of coniferyl alcohol, is largely responsible for the first important understanding of lignin chemistry (Laurichesse et al., 2014). Lignin is a complex natural aromatic heteropolymer (phenolic in nature) that forms long-term carbon flow in the environment. It is the second most abundant (after the sugar polymer cellulose) and complicated natural aromatic heteropolymer (phenolic in nature). The biosphere is believed to have 31011 tons of lignin, which accounts for 30% of the earth's non-fossil organic carbon and is produced at a pace of around 21010 tons each year (Funaoka, 2013; Picart et al., 2015).

Lignin's chemical structure

Lignin is a cross-linked aromatic macromolecule that is formed by the oxidative coupling of monolignols. The three primary monolignols are p-coumaryl, coniferyl, and synapyl alcohols, and their phenylpropanoid monomeric units are p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), respectively (Isikgor et al., 2015; Pollegioni, 2015). Softwood lignin (gymnosperms, conifers like spruce, cedar, and hemlock) contains mostly guaiacyl units with very low levels of p-hydroxyphenyl units (G:S:H = 96:trace:4), whereas hardwood lignin (angiosperms, deciduous trees like poplar, willow, birch, and alder) contains almost equal amounts of guaiacy Grasses (monocots) have G:S:H = 70:25:5 and contain all three units (Wong, 2009). Lignin's molecular weight and linking motifs vary greatly depending on plant species and environmental conditions. Lignification is the cross-linking of lignin monomers or polymer-polymer coupling via oxidases' radicals: resonance, delocalization of radicals to pair at different places generates an array of units linked by carbon-carbon and carbon–oxygen (ether) bonds. -O-4, phenylcoumarane (-5), resinol (-), biphenyl (5-5), 4-O-5 (diaryl ether), and diarylpropane (-1) couplings are the most common bonds or linkages (Isikgor et al., 2015; Pollegioni, 2015).

Lignin Types

For the isolation of lignin, there are no conventional procedures available. Technical lignin and lab scale lignin are available based on lignin isolation technologies. In lignocellulosic refineries, technical lignin is isolated as a byproduct, such as kraft, soda, organosolv, acidolysis lignins, and lignosulphonates (Vishtal et al., 2011). Milled wood lignin (MEL), cellulolytic enzyme lignin (CEL), enzymatic mild acidolysis lignin (EMAL), steam explosion lignin, and pyrolysis lignin are some of the different kinds of lignin (Lange et al., 2013). Because lignins come from a variety of sources and are separated in different ways, they differ significantly from one another. Thermochemical conversion, such as pyrolysis, has gotten a lot of press recently. Biological lignin transformation is nearly completely limited to fungal biopulping and biobleaching. Furthermore, as a byproduct of cellulosic ethanol production, several pretreatment procedures, such as the ethanol organosolv process, can offer high purity lignin (Pan et al., 2006; Alvira et al., 2010; Kosa et al., 2012).

The role of the organism and its enzymatic machinery in the breakdown of lignin

One of the most significant challenges in producing the desired output is the intricacy and differences in lignin structure. Physical and chemical technology (thermochemical treatments, homogeneous and heterogeneous catalysis) typically produces a mixture of undefined products in low yields that are difficult to purify and upgrade (Ragauskas et al., 2014). In the subject of lignin valorisation, biological degradation processes may lead to ecologically benign and selective lignin utilization methods (provided that optimized processes are setup). White-rot fungi, brown-rot fungi, and bacteria have all been found to breakdown lignin using various enzymes and catabolic routes. The controlled enzymatic depolymerization of lignin is a somewhat undeveloped research topic (Picart et al., 2015). Laccase (Lac), lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidases (VPs), protocatechuate-3,4 dioxygenase, aryl alcohol oxidase (AAO), and glyoxal oxidase (GLOX) are some of the major and accessory enzymes that have been identified to modify/degrade lignin (Leonowicz et al., 2001; Wong, 2009).

In comparison to non-phenolic substrates, LiP catalyzes the oxidation of phenolic compounds more selectively and at a significantly faster pace. In the presence of oxygen, phenolic substrates are transformed to phenoxy radicals, which then react with phenolic and non-phenolic moieties to create ring-cleavage products, primarily via C-C cleavage (Higuchi, 2004; Higuchi, 2006; Wong, 2009). MnP may directly oxidize and cleave phenolic structures of lignin via the Mn(III)-chelator complex, however a second mediator is required for non-phenolic lignin moiety. The MnP act via a Mn(III)-chelator complex, which involves 1e-oxidation of the substrate to form a phenoxy radical intermediate, which proceeds through a series of rearrangements, bond cleavages, and nonenzymatic degradation to yield a variety of breakdown products (Wong, 2009). MnP catalyzes C-C cleavage, C-oxidation, and alkyl-aryl cleavages of phenolic -1 and -O-4 lignin substructures, as well as oxidizes phenolic compounds (Higuchi, 2004). Laccases also have the ability to accelerate the direct oxidation of phenolic lignin components. While oxidizing non-phenolic model compounds and -1 lignin dimers in the presence of a mediator (low molecular weight compounds), the degradation of phenolic -1 lignin substructure compounds occurs via the formation of phenoxy radicals, which leads to C-C cleavage, C oxidation, alkyl-aryl cleavage, and aromatic ring cleavage (Higuchi, 2004; Wong, 2009). (1) homolytic or heterolytic breakage of side chains (C-C, alkyl-phenyl) and aromatic rings, (2) O2 attack on carbon-centered radical intermediates, and (3) nucleophilic attack on aryl cations and C cations by H2O and R-OH, which leads to the generation of degradation products (Higuchi, 2006).

As a result, lignin-degrading bacteria use various oxidative enzymatic systems in concert with non-enzymatic systems to assist the effective depolymerization and degradation of lignin. All of these enzymes, however, act by nonselective methods based on their intrinsic radical-based mechanisms, generating random lignin depolymerization and likely condensation of liberated monolignols into more recalcitrant and complicated polymers (Leonowicz et al., 2001; Picart et al., 2015).

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.

Alkali (soda) lignin extraction

Wood powder was treated with varying doses of NaOH (0.1 N, 0.2 N, 0.3 N, 0.4 N, and 0.5 N) at 120 oC for 1 hour to remove alkali (soda) lignin. The solid-to-liquid ratio (w/v) was maintained at 1:15. The dark brown fluid was filtered away, and the volume was reduced to 50 mL by concentrating it in an oven. By lowering the pH of the filtrate to 5.5 with 6 M HCl and then adding 3 liters of 95 percent (v/v) ethanol, the dissolved hemicellulose fraction was precipitated. The residual filtrate was concentrated to 20–30 mL and the pH was adjusted to 1.5–2.0 with 6 M HCl after the hemicellulose fraction was removed by gravity filtering. Centrifugation at 10,000 rpm (Remi R-24, India) for 10 minutes precipitated and sedimented the alkali lignin. For further research, the pellet was dried and stored at room temperature (Sun et al., 2012). The 0.3 N NaOH extracted lignin was chosen for research based on the yield of the lignin component.

Fractionation of alkali lignin by successive solvent extraction (SSE)

From alkali lignin extract, five fractions were isolated using the successive solvent extraction (SSE) method. 0.3 N NaOH was used to make an alkali lignin extract of A. nilotica. The upper organic n-hexane layer (HX) was isolated and concentrated under vacuum after 250 mL of alkali lignin extract was extracted three times with n-hexane (25 mL). With 25 mL of diethyl ether, the residual aqueous layer was extracted three times (DE). Vacuum drying was used to separate the DE layer. The remaining aqueous layer was extracted three times with ethyl acetate (EA2) before being extracted with n-BuOH (BU). The remainder was separated into aqueous fractions (AQ2).

Acidic and Hot water lignin extraction

Acetic and sulphuric acid were used to remove acidic lignin. Acidic extraction was done separately in a soxhlet apparatus using a 3.5 percent (v/v) acetic acid aqueous solution, while sulphuric acid was utilized at a concentration of 17 percent (v/v). The solid-to-liquid ratio (w/v) was maintained at 1:15. The mixture was cooked for 3 hours at 70 degrees Celsius under reflux and then filtered. After filtration, the moisture content of the residue was removed in a vacuum oven (Lab Hosp DTC-03, India). To get lignin, the residue was washed with water and then dried overnight at 60 °C (Pouteau et al., 2003). Hot water extraction was performed in an autoclave at 121 °C for 2 hours using deionized water at a solid/liquid ratio of 1:10 (w/v). The dark liquor was filtered and the filtrate was dried. The dried powder was purified using the alkali lignin purification process described in section 3.2.4.2. (Borrega et al., 2011).

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

Degradation of lignin in terms of disappearance of lignin content and decolorization of lignin.

Table -1 shows the decolorization of lignin (Fig.1 and Table 1) as well as the reduction in the amount of alkali-lignin. In HCLN, F10 and APF4 decolorize lignin 40.16 and 41.68 percent, respectively, however in the HNLC medium, F10 treated lignin decolorizes at a higher rate (24.93 percent) than APF4 treated lignin (19.15 percent).

Table 1: Decolorization (%) during the degradation of lignin in two differentmedium by *E. nidulans* (APF4) and *A. flavus* (F10).

Days	HCLN	HCLN	HCLN	HNLC	HNLC	HNLC
	Control	APF4	F10	Control	APF4	F10
0	0.00	0	0	0.00	0	0
3	0.30	3.04	2.85	0.30	0.47	2.57
6	0.42	6.80	8.30	0.46	2.86	11.58
9	0.20	10.96	13.50	0.64	6.08	14.07
12	1.22	18.62	18.12	1.21	9.22	17.27
15	0.36	30.71	25.52	0.16	12.32	18.87
18	0.51	34.48	31.40	0.48	15.73	23.79
21	0.57	41.67	40.15	0.57	19.16	24.94

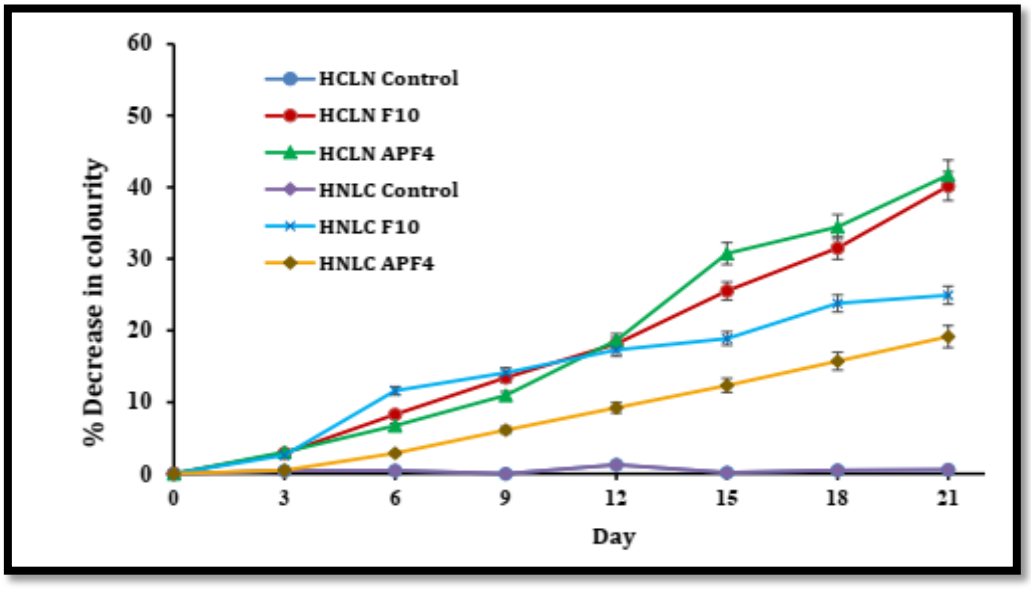

Fig. 1: Decolorization (%) during lignin degradation in control and treated samples.

All of the treated mediums showed considerable alkali-lignin loss. After 21 days of incubation in HCLN medium, the alkali-lignin level was reduced by up to 21% in both mediums, whereas in HNLC it was 19.1 and 14.4% for F10 and APF4 respectively. Both fungi efficiently breakdown and decolorize alkali lignin under HCLN conditions in this investigation.

Table -2: Recovery of lignin content after 21 days.

Sample	Recovered lignin (for 100 mL medium)
HCLN Control	0.243 ± 0.019g
HCLN F10	0.190 ± 0.015g
HCLN APF4	0.192 ± 0.024 g
HNLC Control	0.2404 ± 0.018 g
HNLC F10	0.2032 ± 0.014 g
HNLC APF4	0.2224 ± 0.016 g

Ligninolytic enzymes activity

The biodegradation of alkali lignin was studied for up to 21 days under two different nutritional circumstances, namely HCLN and HNLC, and with A. flavus (F10) and E. nidulans (APF4). Many earlier research have shown that the kind and concentration of carbon and nitrogen sources play a significant role in regulating the production of ligninolytic enzymes by wood-rotting fungus.

Laccase

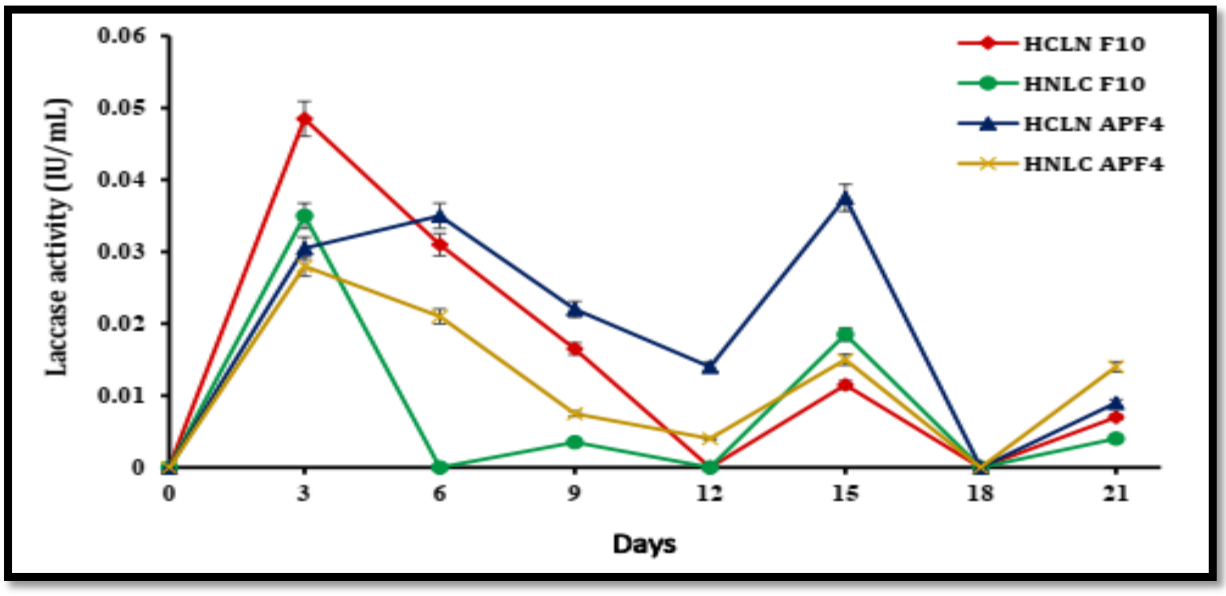

Fig. 2: Laccase activity during lignin degradation under HCLN and HNLC conditions. Data are means of values from duplicate samples; the bars indicate standarderrors.

The formation of laccase was found to be fairly low in all mediums in the current investigation. Laccase activity was initially robust in all four mediums until the third day of incubation, when it began to decline as lignin biodegradation progressed. HCLN F10 (0.05 IU/mL) showed the most action on day 3. The activity in all mediums was very low from day 9 to day 12. From days 12 to 18, the second peak in activity was seen, with the highest activity of laccase (0.04 IU/mL) in HNLC APF4, and then it began to decline. Laccase activity was shown to be greater in HCLN medium compared to HNLC medium in the whole research.

Analysis of degradation products

Quantitative estimation of total phenolic content (TPC) and functionalgroups in lignin fractions (phenolic and carboxyl groups)

Total phenolic levels were also used to measure lignin degradation. When compared to their respective controls, all treated samples had significantly reduced TPC. The quantity of TPC in A. flavus (F10) and E. nidulans (APF4) treated lignin samples was found to be lower under HCLN than under HNLC. TPC levels varied, probably due to changes in ligninolytic enzyme activity under different nutritional circumstances. The findings demonstrated that lignin degradation influenced the TPC of samples under carbon and nitrogen surplus or stress situations. Enzymatic activity was strong under HCLN conditions, causing lignin to depolymerize into tiny fragments and be digested by the fungus.

Table 3: Quantitative determination of polyphenolic content, phenolic hydroxyl group and carboxyl group in control and biomodified lignin samples

samples	**content[1]**	**group (w/w %)**	**group (% w/w)**
HCLN Control	362.20 ± 8.40	1.25 ± 0.03	8.89 ± 0.14
HCLN F10	235.77 ± 12.12 **	1.17 ± 0.02**	9.83 ± 0.12**
HCLN APF4	277.52 ± 6.20 ***	1.24 ± 0.03**	8.24 ± 0.12**
HNLC Control	427.40 ± 10.44	1.09 ± 0.01	10.13 ± 0.12
HNLC F10	320.63 ± 21.12***	1.60 ± 0.04**	10.06 ± 0.12*
HNLC APF4	197.10 ± 8.15 **	1.60 ± 0.03**	9.6 ± 0.10**

The results of functional group analysis using UV spectrophotometric and potentiometric titration methods are reported in Table 4.9. In comparison to their control sample, all treated samples had a high phenolic hydroxyl group, which was confirmed by FTIR analysis. The

phenolic and carboxyl group content of control and biomodified samples were shown to be negatively correlated in our findings.

Analysis of biodegraded and biomodified lignin samples

using gas chromatography-mass spectrometry (GC-MS) GC-MS characterization yielded useful results for the analysis of the aromatic component of the degraded lignin complex. Table 4.10 shows a total ion chromatogram (TIC) of deteriorated alkali lignin fractions extracted with ethyl acetate. The GC-MS study clearly shows that during lignin degradation by F10 and APF4 under two different nutritional conditions (i.e. HCLN and HNLC media), a completely different profile of chemicals was generated. In the ethyl acetate extract of control and biodegraded materials, many low molecular weight aromatic monomeric molecules and acid type compounds were discovered. Specific compounds were also found in nondegraded and degraded lignin samples, which has substantial implications in the fields of pharmaceutics and industry. While ligninolytic bacteria could be a source of production.

The end products of MG breakdown (8 day product) were shown to be non-toxic to the Vigna radiata in this investigation, however the metabolites formed after day 4 of incubation caused toxicity. Partially degraded aromatic compounds and MG were responsible for the toxicity of day 4 metabolites. The MG and partially degraded metabolites had a negative impact on Vigna radiata, as measured by germination index. Overall, the results showed that the lignin degradation fungus A. flavus (F10) has the ability to mineralize MG entirely.

Due to a wide range of possible uses in medicinal, agricultural, optical, and electronic domains, nanoparticles are currently a hot topic of research. Durán et al. (2011) describe nanomedicine as "a leading research focus field that entails synthesizing safe, biocompatible, effective, affordable, and non-toxic medications to tackle diseases." Nanoparticles are prominent in the field of nanomedicine because of their great antibacterial efficacy against a wide spectrum of pathogenic microorganisms and little toxicity to humans (Sintubin et al., 2012; McShan et al., 2014). In this study, lignin-degrading fungi were used as a catalyst for green chemistry-mediated silver nanoparticle (Ag-NPs) synthesis via silver metal ion reduction. For the synthesis of Ag- NPs, the cell lysate was produced and the silver compounds were added. To confirm the synthesis, size, charge, and structure of prepared Ag-NPs, a variety of spectroscopic and analytical approaches were used.

CONCLUSION

The therapeutic potential of the modified lignin samples was investigated in terms of free radical scavenging and anti-diabetic properties. The reducing power, free radical scavenging, reactive oxygen and nitrogen scavenging assays were used to assess free radical scavenging

capacity, while in vitro glucose movement and -amylase inhibition were used to assess antidiabetic potential. The reduction power of lignin samples changed under the HNLC condition was found to be lower. The partial depolymerization of lignin polymer into low electropositive products could explain the decrease in reducing power activity. Nonetheless, the alkali lignin (modified and unmodified) examined in this work has considerable reducing power in the range of 0.4–1 mg/mL, whereas Sultana et al. (2007) found reducing power in the range of 10 mg/mL in the ethanolic extract of A. nilotica bark. Enzymatic production and characterisation of the isolated fungus were also tested. In three different specified media, Minimal Salt Medium broth (MSM), Malt Extract broth (ME), and MSM+ME, A. flavus strains (F10) and E. nidulans (APF4) were screened for ligninolytic enzyme. The A. flavus strains (F10) were chosen for MnP production and characterisation, whereas E. nidulans (APF4) was chosen for Lac production and characterization. MnP was reported to be produced up to 6 U/mL in ME medium, but Lac was found to be 0.1 U/mL in MSM without any inducer. To boost the production of both enzymes, several phenolic substances (2,5 Xylidine, hydroquinone, vanillin, vanillic acid, catechol, ferulic acid, 2,6 dimethoxy phenol) and metal inducers (Cu2+ and Mn2+) were utilized. MnP production was boosted up to 3 times in ME medium by using vanillin and hydroquinone (0.25 and 0.5 mM), whereas Lac production was increased up to 10 times in ME+MSM medium by using 2,5 Xylidine and CuSO4 (0.5 mM). Ammonium sulphate precipitation and ion exchange chromatography were used to purify the enzyme (through DEAE-cellulose). For MnP and Lac, the recoverable fold purification was up to 4.43 and 5.12, respectively, with specific activity of 3.64 and 4.16. To determine the molecular weight, an SDS-PAGE examination was done. MnP has a molecular weight of 42 kDa, while Lac has a molecular weight of 67 kDa.

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