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Value added products from agrowaste

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Abstract

India generates over 400 million tones of agro-forest residues. Agricultural and forest biomass are available in large enough quantities to be considered for large-scale production of alcohol-based fuels. Chhattisgarh is considered rice bowl of India. Rice is an annual crop and one of the byproduct is rice straw. Rice straw can be used as the source of biomasss and lignin.The various other non-woods available are wheat straw, rice straw, barley straw, grass seed straw, flax coil seed, corn stalks, sorghum stalks, sugarcane bagasse, reeds, hemp fiber, sabai grass, cotton staples, stem fibers (hemp, kenaf, jute) etc .Technologies based on the efficient conversion of low quality or waste lignocellulosic residues into fuel and industrially important chemicals represent possible long-term solution to a number of major global problems. The farmers will be benefited economically as their agricultural by-products will provide better cost sharing. The cost of production of chemicals will also be reduced due to involvement of microorganisms and biochemical processes involved therein. The lignin and cellulose obtained from agrowaste can provide a major carbon resource for biotransformation technologies and are anticipated to provide a novel avenue for non-polluting industries by reducing carbon emission.Lignin, as the most abundant natural aromatic material, is being considered for new economical applications such as bio-fuel, binder, dispersant or emulsifier, phenolic resins, carbon fibers automotive brakes, wood panel products, polyurethane foams, epoxy resins for printed circuit boards etc.Various methods, for example, chemical, biological, photochemical, and electrochemical methods, have been explored for the oxidation of lignin to obtain value-added products.The biotransformation of extracted lignin was observed using microorganism. The spectrophotometric analysis between control and experimental samples showed new peaks in region 230 nm and 280 nm in experimental extract. Further analysis of degraded lignin from selected fungus was done by LC-MS. LC– MS (liquid chromatography-mass spectroscopy) was performed with lignin samples obtained from untreated lignin sample as control and treated lignin sample with microorganism. While the lignin sample which was treated with microorganismsshows several new peaks which indicate the degradation of lignin.In long termagrowaste based processes will lead to development of novel cost-effective process and bio-products of industrial importance involving the rural people, academia and industries on a cost-sharing basis thus leading to a sustainable development.

Keywords: Lignin, Biotransformation, Lignin peroxidase, Manganese Peroxidase, UV-Spectrum, LC– MS (liquid chromatography-mass spectroscopy)

INTRODUCTION

The agricultural operations produce large amount of byproducts that have low economical value viz. rice straw, wheat straw, husk, stumps, barley straw, grass seed straw, flax coil seed, corn stalks, sorghum stalks, sugarcane bagasse, reeds, hemp fiber, sabai grass, cotton staples, stem fibers (hemp, kenaf, jute) etc . Some of the waste is utilized as fertlizer, fodder or as fuel.A large quantity remains improperly utilized. A strategy is required to convert this waste into value added products. The process for this conversion should be technologically efficient, economical and with ease of operation. So that a common farmer should be able to optimally utilize its resources to generate secondary source of income.

Lignocellulose is a class of biomass that consists of three major compounds cellulose, hemicellulose and lignin. It also includes water and a small amount of proteins and other compounds, which do not participate significantly in forming the structure of the material (Raven et al., 1992). Inside the lignocellulose complex, cellulose retains the crystalline fibrous structure and it appears to be the core of the complex. Hemicellulose is positioned both between the micro- and the macrofibrils of cellulose. Lignin provides a structural role of the matrix in which cellulose and hemicellulose is embedded. Considering that cellulose is the main material of the plant cell walls, most of the lignin is found in the interfibrous area, whereas a smaller part can also be located on the cell surface (Faulon et al., 1994).

Lignins are polyphenols characterized by a complex network derived via the free radical polymerization of the three main monomeric phenyl propanonic units, p-coumaryl ,coniferyl and sinapyl alcohols bonded through an array of different interunitbondings. In plant lignin synthesis occurs through lignification process. Lignification is polymerization process of the monomeric polyphenol, which occur in the cell wall to produce radical and couple them with other monomeric radicals. Bond formation between the monomeric polyphenol is through oxidative (radical-mediated) coupling, which give rise to lignin polymer/oligomer and finally build up to a phenylpropanoid polymer. The oxidative coupling between monomeric polyphenol can result in

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the formation of several different interunit linkages (Hatfield, 2001).It is a cross-linked racemic macromolecule with molecular masses in excess of 10,000 u. It is relatively hydrophobic and aromatic in nature. It is one of the most abundant organic polymers on Earth, exceeded only by cellulose, employing 30% of non-fossil organic carbon and constituting from a quarter to a third of the dry mass of wood. Its most commonly noted function is the support through strengthening of wood (xylem cells) in trees (Argyropoulos and Menachem, 1997).

Lignin plays a significant role in the carbon cycle, sequestering atmospheric carbon into the living tissues of woody perennial vegetation. Lignin is one of the most slowly decomposing components of dead vegetation, contributing a major fraction of the material that becomes shumus as it decomposes (Sarkanen and Ludwig, 1971). The chemical structure of lignin has also been difficult to determine, and even very recently, new bonding patterns have been described in softwood lignin, e.g. Dibenzodioxocin structures. The degree of polymerisation in natureis difficult to measure, since it is fragmented during extraction and the molecule consists of various types of substructures that appear to repeat in a haphazard manner ("Lignin and its Properties",2001).

Several different enzymatic, chemical and mechanical methods have been developed for the isolation of the lignin from wood and pulp. These are-

1. Isolation by Enzymatic Hydrolysis- Yamasaki et al. (1981) first developed a technique for isolating residual lignin from kraft pulp by enzymatic hydrolysis. This procedure is based on selective hydrolysis and dissolution of carbohydrates in pulp by cellulolytic enzymes, leaving lignin behind as an insoluble residue.
2. Isolation by Acid Hydrolysis-This method involves the extraction of lignin via acidic treatment. Prior to this, kraft pulps are typically extracted with acetone to remove extractives. The extracted pulp is then refluxed with 0.1 M HCl in 9:1 dioxane:water. The solubilized lignin is then recovered from solution (Froass*et al.*1996).
3. Isolation by Alkaline Hydrolysis- It is based on saponification of intermolecular ester bonds crosslinking xylan hemicellulose and other components (Sun and Cheng, 2002). It can be done by using either Calcium or sodium hydroxide or Ammonia. Process conditions are relatively mild but reaction times can be long. These mild conditions prevent condensation of lignin, resulting in high lignin solubility (Chang and Holtzapple, 2000).
4. Isolation by Organic Solvents-This method uses single or mixture of organic solvents, ex. Mixture of pyridine, acetic acid and water in the ratio 9:4:1 (v/v/v) has been used to extract lignin. This method is used to isolate water soluble lignin in the form of lignin carbohydrate complex. This is followed by extraction with chloroform. The aqueous layer is then dialyzed against water and then lyophilized. Lyophilized material is then precipitated by ether to remove low molecular weight compounds (Gullischen and Fogelholm, 2000)

Lignin is both the most abundant aromatic (phenolic) polymer and the second most abundant raw material. It is degraded and modified by microbes in the natural world.Biodegradation of lignin is a prerequisite for processing biofuel from plant raw materials. Lignin is indigestible by animal enzymes, but some fungi (such as the Dryad's saddle)are able to secrete ligninases (also named lignases) that can biodegrade the polymer. The enzymes involved employ free radicals for depolymerization reactions (Carlile*et al.,* 1994). Well understood lignolytic enzymes are manganese peroxidase, lignin peroxidase and cellobiose dehydrogenase. Furthermore, because of its cross-linking with the other cell wall components, it minimizes the accessibility of cellulose and hemicellulose to microbial enzymes. Hence, in general lignin is associated with reduced digestibility of the overall plant biomass, which helps defend against pathogens and pests (Sarkanen,1971).

Microbial degradation of lignin (Martínez et al., 2005; Kersten and Cullen, 2007) represents a key step for closing the carbon cycle, since removal of the lignin barrier enabled the subsequent use of plant carbohydrates by other microorganisms. Lignin removal is also a central aspect in industrial uses of cellulosic biomass, such as bioethanol production and manufacture of cellulose-based chemicals and materials, including paper cellulose pulp manufacture basically consists in breaking down (chemically or mechanically) the middle lamella in such a way that wood fibers are individualized (Sixta, 2006). In the industrial applications, biotechnology based on lignin-degrading microbes and their enzymes can contribute to more efficient and environmentally sound use of renewable lignocellulosicfeedstocks for sustainable production of materials, chemicals, biofuels and energy.

Material and Method

Sample Collection: Acacia wood dust (mash size 120) was obtained from local wood mill inBilaspur, Chhattisgarh.

Pretreatment of materials: Acacia wood dust was dried overnight at 60°C for to remove the moisture. The Acacia wood dust (Approx 90g) was treated with acetone-water (9:1, v/v) mixture in a soxhlet apparatus at 56 ºC for 6-8 hrs for the removal of lipid and waxes. The treated material was dried at the room temperature and was further used for extraction of lignin.

Extraction Method

1. Alkaline Extraction Method:-15g wood dust of Acacia was boiled in solution of (0.5N, 1N, 1.5N, 2N) NaOH (10ml NaOH/gm of wood dust) for 2 hours and solution was filtered through filter paper. The filtrate was concentrated to 75ml by keeping in hot air oven at 60ºC in order to get lignin carbohydrate complex (Lawther 1996).
2. Hot Water Extraction:-Dried powdered material of wood dust of acacia was stirred with distilled water (30ml/g) and autoclaved for 20 min at 120 ºC, the filtrate was dried in oven.(Tilquin, 2002).
3. Solvent extraction:- Milled Wood dust of Acacia was dissolved in pyridine: acetic acid: water (9:1:4 v/v/v) and the above solution was extracted with chloroform in a separating funnel which separates two layers i.e. aqueous layer and chloroform layer. The aqueous layer containing the lignin carbohydrate complex was dialyzed against water by using dialysis membrane and dipped in water and was kept overnight in magnetic stirrer. Dried the dialyzed aqueous layer to obtain lignin carbohydrate extract (Gullischen and Fogelholm, 2000).

Characterization of lignin
Polyphenol content

Total phenolic compounds were determined by reaction with phosphomolybdotungstate acid (Singleton and Orthofer, 1999) with slight modification. 1ml of each extract (150 µg/ml) was added to 0.5 ml of Folin-Ciocalteu phenol reagent (Merck Pvt. Ltd., India) and 1ml of 20% (w/v) sodium carbonate makeup the volume up to 5ml with distilled water. The mixture was allowed to stand at room temperature for 10 min. The resulting blue complex was then measured at 760 nm against blank. All the observations were in three replicates for statistical analysis.

Carbohydrate Impurity

Sample was diluted 500 fold and 1 ml sample was addedin 4 ml Anthrone reagent. Mixture was kept in boiling water bath for 8 minutes and then cools to room temperature. O.D. was taken at 630 nm. (Sadashivam and Manickam,2008)

Lignin fractions were further characterized based on UV-visible spectrophotometry and FTIR.UV spectra were recorded on a UV-visible spectrophotometer model- Shimadzu UV-1800 Double Beam. The samples from the inoculated lignin broths (0.25% w/v alkali lignin) were diluted to 50 times and then spectrum was taken. Uninoculated lignin broth (0.25% w/v alkali lignin) was used as control and for baseline correction for taking the UV spectrum of the inoculated samples.

Infrared spectra was recorded on a, FTIR Spectroscopy Shimadzu using KBrpowder. Lignin (1mg) extracted from different methods were mixed and thoroughly ground with 300mg of KBr to reduce particle size and to obtain uniform dispersion of the sample in the disks. The frequency range was 700nm-40000cm^{-1}.

Biodegradation of lignin

Phanerochaetechrysosporium (MTCC no.787) inoculated in lignin containing minimal media (alkali lignin 0.25% w/v) was incubated for 15 days at 120rpm and 28ºC.2ml Sample was centrifuge at 3600 rpm. Supernatant obtained were characterizedby thin layer chromatography (TLC) and mass analysis.

To detect the degradation product TLC was performed using n-hexane, chlorobenzene and glacial acetic acid (31:14:5) as solvent. The chamber was presaturated with corresponding solvent and sample of extracted lignin and degraded lignin (50µl) was loaded 1cm up from the bottom of silica G plate.

Mass spectrometry was used for determination of molecular weight and putative identification of degradation products.. Mass analysis was in positive ion mode. The mass spectrums of the samples were recorded on the Mass Spectrophotometer model- LC-MS/MS Applied Biosystems API 2000, with conditions of declustering Potential (DP)- 80, Focusing Potential (FP)- 400 and Entrance Potential (EP)- 10. Samples were prepared inHPLC grade ethanol water mixture (1.5 ml HPLC grade ethanol and 1.5 ml HPLC grade distilled water were mixed and then 33 µl of sample was added to it). Sample was injected in the mass analyzer with the flow rate of 50 µl/min.

RESULTS AND DISSCUSION

Overnight dried sample were subjected to remove the lipid portion present in it. Then three different extraction methods were used out of these alkali method gives highest amount of lignin as compare to hot water and solvent water extraction.Total yield of lignin from different extraction methods are alkali extraction (0.5N NaOH) 188 g / kg *Acacia* wood dust, hot water extraction 56g / kg *Acacia* wood dust and solvent extraction 32 g / kg *Acacia* wood dust.

The Anthrone test was performed to test the presence of carbohydrates in all the extracted samples and the result was found to be negative.And the Folin Denis test was performed for the presence of polyphenol content. All extracted sample show positive result for the presence of polyphenols. These two result indicate that the extracted lignin were free from carbohydrate impurity and the sample contain only polyphenolic content.

UV-visible spectrophotometry has been used to study for the characterization of extracted samples. All extracted lignin (alkaline, hot water and solvent) fractions illustrate the basic style of lignin, which have a maximum absorption coefficient at about 270 nm, originating from phenolic hydroxyls conjugated to α-carbonyl, carbon-carbon double bonds, or biphenyl groups in lignin. The absorption at 320 nm is due to the small quantity of the associated hydroxycinnamic acids (p-coumaric and ferulic acids).

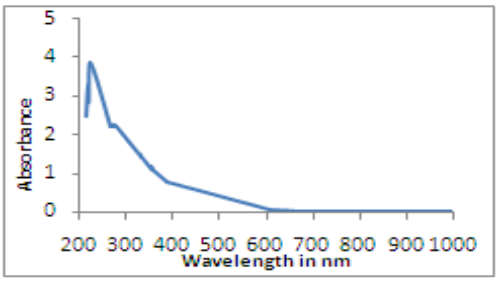

Fig 1. UV- Vis Spectrum of Hot water extracted lignin

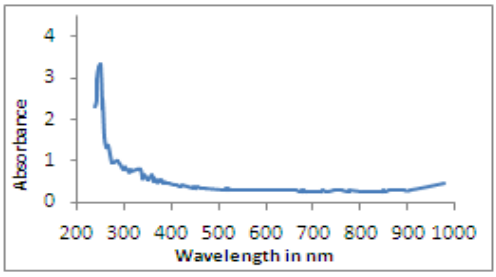

Fig 2.UV- Vis Spectrum of Alkali (0.5N NaOH) extracted lignin

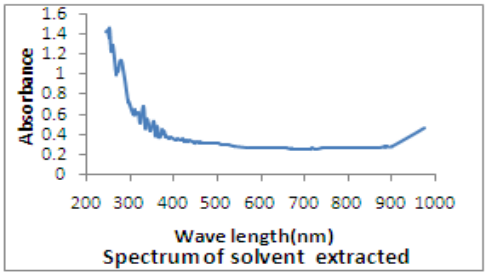

Fig 3.UV-Vis Spectrum of Solvent Extracted Lignin

FTIR analysis of different extracted product shows different corresponding peak at different wavelength region. Following table 1 shows the detail of functional groups, side chain of functional groups, bending, stretching and vibrational description and their corresponding peaks.

Table1. FTIR analysis of different extracted lignin

Alkali extracted lignin	Solvent extracted lignin	Hot water extracted Lignin	Functional groups description
3410	3412	3248	O–H stretch, H-bonded
2935	2941	2947	C–H stretch methyl and methylene groups
-	-	2870	C–H stretch in methyl and methylene groups
-	-	2835	C–H stretch O–CH_3 groups
1728	1722	-	C=O stretch, unconjugated ketone, carboxyl, and ester groups
1680	-	1691	ring conjugate C=O stretch of coniferaldehyde/sinapaldehyde
1631	-	-	ring conjugated C=C stretch of coniferyl/sinapyl alcohol
1600	1606	1604	aryl ring stretching, symmetric
1512	1529	1514	aryl ring stretching, asymmetric
1485	-	1485	C–H deformation, asymmetric
1442	1433	-	O–CH_3, C–H deformation, asymmetric
1382	-	1369	O–CH_3, C–H deformation symmetric
-	1344	1323	Aryl ring breathing with C–O stretch
1278	1282	1278	Aryl ring breathing with C=O stretch
1157	1142	1159	Aromatic C–H in plane deformation
1066	-	1076	C–O deformation, secondary alcohol and aliphatic ether
1037	1035	-	Aromatic C–H in plane deformation
-	-	939	C–H deformation out of plane, aromatic ring
871	867	-	C–H deformation out of plane, aromatic ring
819	842	-	C–H deformation out of plane, aromatic ring
742	-	761	skeletal deformation of aromatic rings, substituent groups, side chains

Agarwal and Ralph (1997a) andAgarwalUmesh*et al.,*(2005)described the contribution of bonding pattern of basic monomer unit of lignin and functional groups in soft and hard wood lignin. The above infrared structure study shows the similar functional groups and banding pattern between lignin monomers. This study also reveal not onlyof inter-phenylpropane-unit linkages of carbon-tocarbonand carbon-to-oxygen, but also of side chainswith various substituent and functional groups.In the above table various different raman peak represent the aliphatic and aromatic C-Hstretches (2800-3100 cm^{-1}), aromatic rings ethylenic C=C, α- and β- C=O (1500-1800 cm^{-1}) and CH_3, CH_2,CH bending modes and its vibration (was shown in 1500-1000 cm^{-1}) (AgarwalUmesh P.,1997b).The aboveinfrared spectrum of lignin indicates the purity of the extracted lignin.

Thin layer chromatography was performed for the determination of degraded product with native extracted product. The Rf value of the sample were show in following table 2.

Table 2.Rf value of different lignin samples

Standard/ sample	R_f
Tannic acid (standard)	0.48
Alkaline extract lignin	0.47
Solvent extracted lignin	0.48
Hot water extracted lignin	0
Lignin broth (inoculated with *Phanerochaetechrysosporium*)	0.45
Lignin broth (control)	0.45

The Rf value of extracted lignin and biodegraded lignin show same Rf with standard (tannic acid), indicate that the extracted sample having same or similar composition with standard.

Following compounds were identified and presented in table 3 by the mass analysis of lignin from the lignin broths inoculated with*Phanerochaetechrysosporium.*

Table 3. Molecular mass analysis of degraded lignin sample with control sample

S. No.	Compound Name	In control broth	In *Phanerochaetechrysosporium*inoculated broth	m/z ratio
1.	Syringic Acid	Present	Present	71
2.	p-coumaric Acid	Absent	Present	71
3.	Salicylic Acid	Present	Present	73
4.	Phenoxyacetic Acid	Present	Present	73
5.	Phenylacetate	Present	Present	73
6.	Catechol	Absent	Present	73
7.	3-methylpyrrole	Present	Present	81
8.	Stryrene	Present	Present	99
9.	Hydroxycinnamic Acid	Absent	Present	99

10.	Benzene acetonitrile	Present	Absent	113
11.	4-hydroxy phenyl acetic Acid nitrile	Absent	Present	135
12.	Phthalate	Present	Present	149
13.	3-(2-hydroxyphenyl propionic acid)	Present	Present	149
14.	Veratraldehyde	Absent	Present	163
15.	Benzoic Acid	Absent	Present	176
16.	p-hydroxybenzaldehyde	Absent	Present	176
17.	2,3,5-trimethoxy toluene	Absent	Present	185
18.	Cinnamic Acid	Present	Present	193
19.	Protocatechuic Acid	Present	Present	193

The chemical properties of the lignocellulosics make them amenable to enormous biotechnological applications. Numerous studies have been carried out to isolate microbes degrading lignin polymer (Ball et al. 1989). Crude lignin is although not-biodegradable anaerobically, yet it is possible to promote only minor modification in functional groups (Alexandreand Zhulin, 2000). The non-lignin compounds and some low-molecular weight materials produced as a result of chemical modifications are, nevertheless, degraded through microbial activities (Hatakka, 1994). The white rot basidiomycetes degrade lignin rapidly and extensively than any other microbial groups (Kent et al., 1987) during which they invade the lumens of wood cells, where they secrete enzymes that degrade lignin and other wood components (Eriksson et al.,1990). Fungal modification of residual lignin in wood generated many useful products derived from lignin units identified as guaiacol , syringol and their derivatives, propylsyringol , several oxidized products (aromatic aldehydes and ketones) such as vanillin, homovanillin, acetoguaiacone, guaiacylacetonepropiovanillone, syringaldehyde, homosyring aldehyde, acetosyringone, syringylacetone, propiosyringone, sinapaldehyde. Major lignin-derived compounds in the degraded wood are syringol, 4-vinylsyringol syringaldehyde trans-4-propenylsyringol, acetosyringone (Del Rı´o et al. 2001; Del Rı´o JC et al. 2002; Martínez et al. 2005). According to the above table it clearly shows that new compound of small molecular weight were formed by the enzymatic activity of Phanerochaetechrysosporium. The formation of various other small molecules is action of ligninolytic enzymes like laccase, lignin peroxidases and manganese peroxidase. Formation of various low molecular compound like syringic acid, p-coumaric acid, Catechol, Veratraldehyde, Benzoic acid, Cinnamic acid indicate that the organism utilize the lignin and form these useful small molecular product. It also shows that the organism also use lignin as a secondary carbon source in extreme condition.

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