

Review

Engineering Plant Biomass Lignin Content and Composition for Biofuels and Bioproducts

Cassie Marie Welker ¹, Vimal Kumar Balasubramanian ¹, Carloalberto Petti ³,
Krishan Mohan Rai ¹, Seth DeBolt ² and Venugopal Mendu ^{1,*}

¹ Fiber and Biopolymer Research Institute, Department of Plant & Soil Science, Texas Tech University, 2802 15th Street, Lubbock, TX 79409, USA; E-Mails: cassie.welker@ttu.edu (C.M.W.); vimal-kumar.balasubramanian@ttu.edu (V.K.B.); krishan.m.raii@ttu.edu (K.M.R.)

² Department of Horticulture, University of Kentucky, 1100 Nicholasville Road, Lexington, KY 40546, USA; E-Mail: sdebo2@uky.edu

³ Department of Science and Health, Carlow Institute of Technology, Kilkenny Rd, Carlow R93V960, Ireland; E-Mail: carloalberto_petti@outlook.com

* Author to whom correspondence should be addressed; E-Mail: venugopal.mendu@ttu.edu; Tel.: +1-806-632-7929.

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Abstract: Lignin is an aromatic biopolymer involved in providing structural support to plant cell walls. Compared to the other cell wall polymers, *i.e.*, cellulose and hemicelluloses, lignin has been considered a hindrance in cellulosic bioethanol production due to the complexity involved in its separation from other polymers of various biomass feedstocks. Nevertheless, lignin is a potential source of valuable aromatic chemical compounds and upgradable building blocks. Though the biosynthetic pathway of lignin has been elucidated in great detail, the random nature of the polymerization (free radical coupling) process poses challenges for its depolymerization into valuable bioproducts. The absence of specific methodologies for lignin degradation represents an important opportunity for research and development. This review highlights research development in lignin biosynthesis, lignin genetic engineering and different biological and chemical means of depolymerization used to convert lignin into biofuels and bioproducts.

Keywords: plant cell wall; lignocellulose; lignin; depolymerization; genetic engineering; degradation; pyrolysis; hydrogenolysis

1. Introduction

Chemical resources are in continuous demand by developed and developing countries alike, and it is estimated that there will be a 50% increase in consumption of plant resources for food and fuel by 2050 [1,2]. Coupled with this is the growing demand for cleaner and renewable sources of chemicals for fuels and products. Currently, the energy demand is met by the use of fossil fuel, a non-renewable energy source, which results in an increase in greenhouse gas emissions. Alternatively, solar, wind, hydroelectric and biomass could stand as eminent sources for producing renewable energy, while reducing the amount of greenhouse gases produced by petroleum based resources. Biomass (wood residues and pulping liquor)-derived energy accounts for more than the combined amount produced from hydroelectric and geothermal sources in the United States as of 2005 [3]. Also, only biomass retains the capacity to produce liquid biofuels and byproducts. The word biofuel encompasses a variety of transportation fuels such as bioethanol, butanol, biooil and biodiesel. Though all of these biofuels are derived from plant material, the source of raw material (biomass or seeds) and the production processes are different. Bioethanol is derived from converting the depolymerized starch, sugars or lignocellulosic material into ethanol by fermentation process. Biooil is produced by pyrolysis of whole plant biomass, particularly high-lignin biomass, to produce energy dense fuels [4], while biodiesel is produced by the transesterification of vegetable oil or other lipids [5].

The first-generation biofuels were produced from food crop materials such as corn, sugarcane, and palm oil which led to a food/fuel competition [6]. Using food crops for biofuel production resulted in an increase in the price of food grains and other allied products [7]. The use of farm produce for biofuels is difficult to justify whilst malnutrition is still a major problem in the world [7,8]. The food/fuel competition resulted in the exploration of alternative feedstocks for biofuel production *i.e.*, lignocellulosic material. Lignocellulosic components of plant cell walls, like cellulose, hemicellulose and lignin [9], represent an interesting and challenging opportunity for producing cleaner and renewable liquid fuels. Utilization of lignocellulosic material for biofuel and biochemical production could greatly reduce CO₂ emissions [10,11]. In order to meet the worldwide energy demand, increased focus on efficient plant breeding, biotechnology and process development will be needed along with collaboration between the fuel and energy sectors [5,12–14].

2. Lignocellulosic Biomass, the Focus of Second-Generation Biofuels

Feedstock, in the context of the biofuel industry, represents any biomass that is used as a raw material for the production of biofuels. Plant organs such as fruits, seeds, and tubers, from various crops such as rapeseed, corn, sunflower, soybean, cotton and palm that store simpler starch and lipids, were used for first-generation ethanol and biodiesel production (Figure 1). Alternatively, the entire above-ground plant biomass known as lignocellulosic feedstock is used for the production of second-generation biofuels.

The term lignocellulosic feedstocks refer to the inedible stems and leaves of the above-ground plant body. Dedicated biofuel crops such as switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus spp.*), sorghum (*Sorghum spp.*) and eucalyptus (*Eucalyptus spp.*) are grown, and some of these crops are adapted to poor soils and marginal agricultural lands [15], reducing the land use competition for food crops (Figure 1). Apart from dedicated biofuel crops, there are other sources of lignocellulosic materials such as agricultural and industrial waste. Plant organs that are unsuitable for human consumption are mostly considered agricultural waste. The dried stalk (straw) of grain crops such as maize, soja, rice, wheat, yucca and barley are the main sources of agricultural waste that can be used for the production of biofuels. Though this type of agricultural waste represents a great source of lignocellulosic material, pelleting and transportation are major concerns.

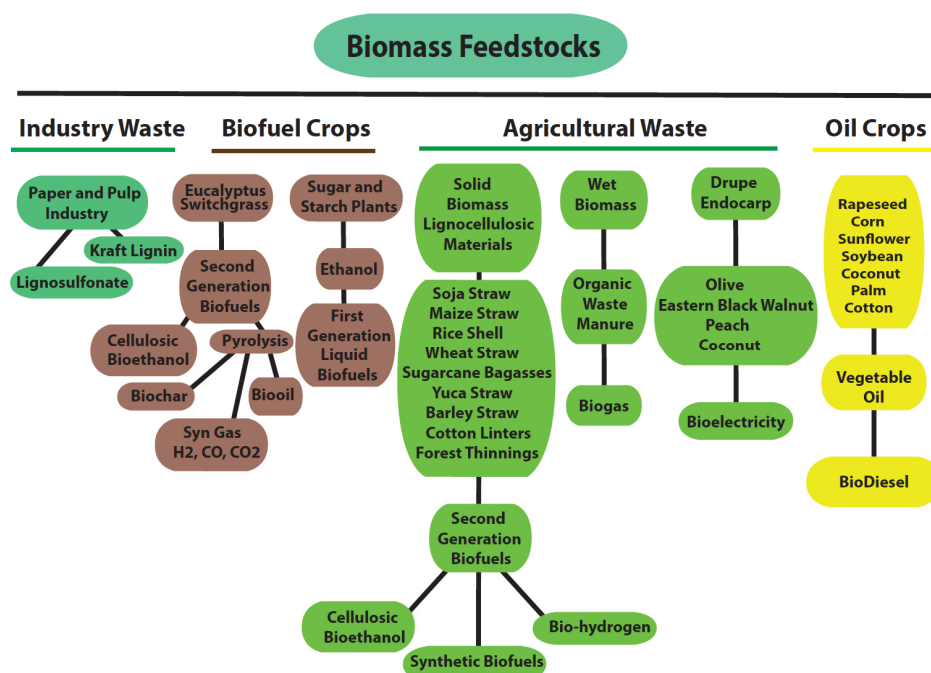


Figure 1. Biomass feedstocks and their utilization in the production of biofuels, bioenergy and bioproducts. The figure includes the first- and second-generation feedstocks and their utilization for different bioproducts (liquid biofuels, biochar, bioelectricity and biogas).

High density feedstocks such as the drupe fruit endocarps (shells) of olives, eastern black walnut and coconut have the highest lignin content of all known plant organs, and the energy derived from the endocarp is comparable to coal [16]. The endocarp is an inedible portion of an edible fruit, and therefore, can be considered for dual purposes: food and fuel. Coconut shell, for example, has no use as a human or animal feed and is not reintegrated into the soil. The fruit endocarp material has high energy density due to its high-lignin content and can be an excellent feedstock for the production of bioelectricity by gasification or high-energy dense biooil via pyrolysis. There are about 24 to 31 million tons of drupe endocarp biomass available in the world [10], which is highly underutilized, and a recent assessment suggests that proper utilization could benefit the countries with energy scarcity [16]. Additionally, other forms of agricultural (forest thinnings, cotton linters) and industrial wastes (sugarcane bagasse, paper and pulping waste) could be used as lignocellulosic feedstocks. Apart from dry wastes, wet biomass can also be utilized for the production of biogas, a mixture of mainly carbon dioxide and methane [17].

The cell wall of plant biomass consists of several major polymers: cellulose, hemicellulose, lignin, and minor components such as organic acids, proteins, tannins as well as secondary metabolites of intrinsic value. The composition of lignocellulosic material varies between different species, varieties, plant parts and environmental conditions (Table 1, [16,18]). This is particularly noticed between angiosperm (hardwoods) and gymnosperm (softwoods) plants [18,19]. Apart from the biological and environmental elements, other factors such as harvesting time, method and storage conditions also affect the composition of the feedstocks [20]. The variability of the lignocellulosic material creates problems in selecting or developing efficient processing methods [21].

Bioethanol production from cellulose is plagued by the pretreatment costs of the biomass to partially separate cellulose from lignin and to allow for enzymatic hydrolysis. Here, the presence of lignin hinders the microbial enzymatic reaction due to its complex structure and inhibitory properties on sugar and ion transport systems in the microbial cell membranes [22]. Although lignin's richness in aromatic compounds is beneficial for bioproduct industries, there is difficulty in finding a catalyst to cleave the random chemical bonds present in lignin without repolymerization [19,23]. A complete understanding of the lignin biosynthetic pathway, including the underlying regulatory mechanisms, is essential for efficiently engineering and harnessing the energy stored in lignocellulosic material.

3. Lignin Biosynthesis and Composition

Lignin is the second most abundant polymer present in lignocellulosic material, and it is deposited in the walls of certain specialized plant cells, which undergo secondary cell wall biosynthesis. Apart from providing mechanical support to the sap-conducting xylem cells, lignin also plays an important role in plant defense, in seed dispersal and in the formation of an apoplastic diffusion barrier in the roots [24–27]. The existence of lignin in the cell wall of land plants has contributed to their capacity to conquer terrestrial environments and to protect themselves against various biotic and abiotic stresses [28,29]. Structurally, lignin is a complex heteropolymer consisting of three main canonical units, namely, *p*-coumaryl (H), coniferyl (G), and sinapyl (S) monomers [30], which are derived from the phenylpropanoid pathway. The monomers vary in the degree of methoxylation of the aromatic ring at the *ortho*-, *meta*- and *para*-positions [31]. Phenylalanine, the primary substrate in the lignin biosynthetic pathway, is derived from the shikimate pathway and then modified by the enzyme phenylalanine ammonia lyase (PAL) to form cinnamic acid. Subsequently, a series of enzymes such as hydroxylases [*p*-coumarate-3-hydroxylase (C3H), cinnamate-4-hydroxylase (C4H), ferulate-5-hydroxylase (F5H)], methyl transferases [caffeic acid-O-methyltransferase (COMT), caffeoyl-CoA-O-methyltransferase (CCoAOMT)], reductases [cinnamoyl-CoA-reductase (CCR)], and dehydrogenases [cinnamyl alcohol dehydrogenase (CAD), sinapyl alcohol dehydrogenase (SAD)], produce lignin biosynthetic pathway components such as alcohols, acids and aldehydes [30]. These components are utilized for the lignin biosynthesis and for the production of other aromatic compounds. The biosynthetic pathways leading to naringenin, flavonoid and anthocyanins (flavor and color compounds of considerable economic importance) on one side and lignin on the other side, have been elsewhere reviewed [30].

Table 1. Lignin variation between systematic divisions and species.

Division	Lignin Occurrence and Composition	Interspecies Variation	Cell Wall Composition (%)			References
			Cellulose	Hemicellulose	Lignin	
Algae	Vary in the composition of G, H and S subunits over time	<p>Though lignin is not present in algae, some exceptions exist:</p> <ul style="list-style-type: none"> ▪ In <i>Calliarthron cheilosporioides</i>, S subunit of lignin is found ▪ In some sea weeds, compounds resembling lignin are found 	~70	~43	NA	[32–36]
Bryophytes	Mostly intermediate compounds like 1,3,5-hydroxybenzene	<p>Most bryophytes lack lignin</p> <ul style="list-style-type: none"> ▪ In <i>Dawsonia sp.</i> and <i>Dendroligotrichum sp.</i>, 6%–10% of gametophyte axes contain lignin like compounds 	NA	NA	NA	[32,37,38]
Pteridophytes	Present in the vascular system (tracheary elements). Mostly G and H subunits and rarely S subunits	<p>High variation is seen in S subunit composition:</p> <ul style="list-style-type: none"> ▪ In <i>Equisetum silvaticum</i>, up to 10% S subunits are present ▪ In <i>Selaginella martensii</i>, 43% of S subunits are present in lignin 	~50	NA	~14	[32,39–41]
Gymnosperms	Present in the vascular system (tracheary elements). Mostly G and H subunits and lesser amounts of S subunits	<p>High variation is seen in S subunit composition:</p> <ul style="list-style-type: none"> ▪ Unlike in stems of <i>Ginkgo biloba</i> containing 1% of S subunits, in suspension cultures up to 11% of S subunits are present ▪ In <i>Ephedra viridis</i>, 60% of S subunits are present in lignin 	NA	NA	~30	[32,39,42]
Angiosperms (Monocots)	Present in vascular system (tracheary element and fibers). Significant amounts of all three G, S and H subunits	<p>Variation is seen in phenolic acid content of lignin:</p> <ul style="list-style-type: none"> ▪ In <i>Maize</i> bran, <i>p</i>-coumarates and ferulates account up to 4% of lignin composition ▪ In some C4 grasses, <i>p</i>-coumarate acrylates accounts 20% of the lignin present 	20–45	20–50	20	[32,43–45]
Angiosperms (Dicots)	Present in vascular system (tracheary element and fibers). Mostly G and S subunits and minor level of H subunits	Varies between 10%–20% in different species	15–50	5–30	7–10	[32,34,45]

NA: data Not Available.

Once synthesized, the monomeric lignin subunits are transported across the plasma membrane into the apoplastic space by transporters. Thus far one transporter, the adenosine triphosphate (ATP)-binding cassette (ABC) transporter, has been reported to be involved in *para*-coumaryl alcohol transport [46]. The transporters involved in the other two monolignols have not been discovered. Once exported, the monolignols are converted into free radicals (tautomeric structures) by the action of phenol-oxidizing enzymes such as laccases and peroxidases [47]. These free radicals form the lignin polymer by a random coupling process in the cell wall and middle lamellae of cells undergoing the lignification process. The lignin composition and structure depend on the availability of monomers and the type of bonds formed during the polymerization. The formation of condensed (5-5', β -5', β - β , β -1') or non-condensed bonds (β -O-4', 4-O-5', 5-O-4') depends upon the spatial constraints posed by the surrounding polymers, the availability of lignin monomers and the reactive ends present on the available monomeric subunits [48].

Since these free radicals are produced by the phenol oxidases which have substrate specificity, the expression of phenol oxidases determines the composition of the polymer. Laccases are a large group of phenol oxidizing enzymes with a multitude of functions [49], including free radical formation. Recent study in *Arabidopsis* (*Arabidopsis thaliana*) using Transfer-DNA (T-DNA) mutants, showed a preferential selection of a specific lignin monomer (coumaryl monomer units) by laccase (LAC) 17 in the sclerenchymal cells of developing stems [50]. *Arabidopsis* laccase 4 and 17 are involved in the secondary cell wall lignification of the protoxylem [51]. In *Brachypodium distachyon*, for instance, laccase 5 was shown to be responsible for culm lignification [52], hence the expression of phenol oxidases is important for lignification. Apart from laccases, peroxidases are another class of oxidizing enzymes that act non-redundantly with laccases in the free radical formation [53]. Overall, lignin biosynthesis has several enzymes, transporters and phenol oxidases which determine the final lignin polymer composition. The presence/absence of genes or variation in gene expression largely influences lignin deposition at the cellular level, which is then reflected at a higher organismal level (Table 1, [28]).

3.1. Lignin Composition and Structure Analysis

Though the lignin biosynthetic pathway produces monolignols, the polymerization process depends on the monolignol export, oxidizing enzymes and the availability of monomers in the apoplastic region of the cell. Hence, the exact composition of lignin is highly variable in different cell types, tissues and organs, and therefore, it is essential to know the detailed composition for gene functional studies. This requires compositional and structural analytical methods to study the nature of lignin in different cell types, tissues or organs within a plant. For example the lignin content and composition of coconut shell is different from coconut wood. So, apart from understanding the biosynthetic pathways, it is important to analyze the structure and composition of lignin to have a comprehensive understanding of the lignification process.

Lignin structural analysis requires careful extraction without altering the native structure of the polymer while compositional analysis needs complete breakdown of the polymer into monomers. Degradative techniques such as oxidation, reduction, hydrolysis and acidolysis are used for the lignin compositional analysis, while derivatization techniques such as thioglycolic acid (TGA) and acetyl bromide (ACBR) are used for the structural analysis (Figure 2). The end products obtained from the

degradation of lignin vary based on the lignin composition, and the end products are analyzed by using techniques such as gas chromatography, mass spectroscopy and proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy.

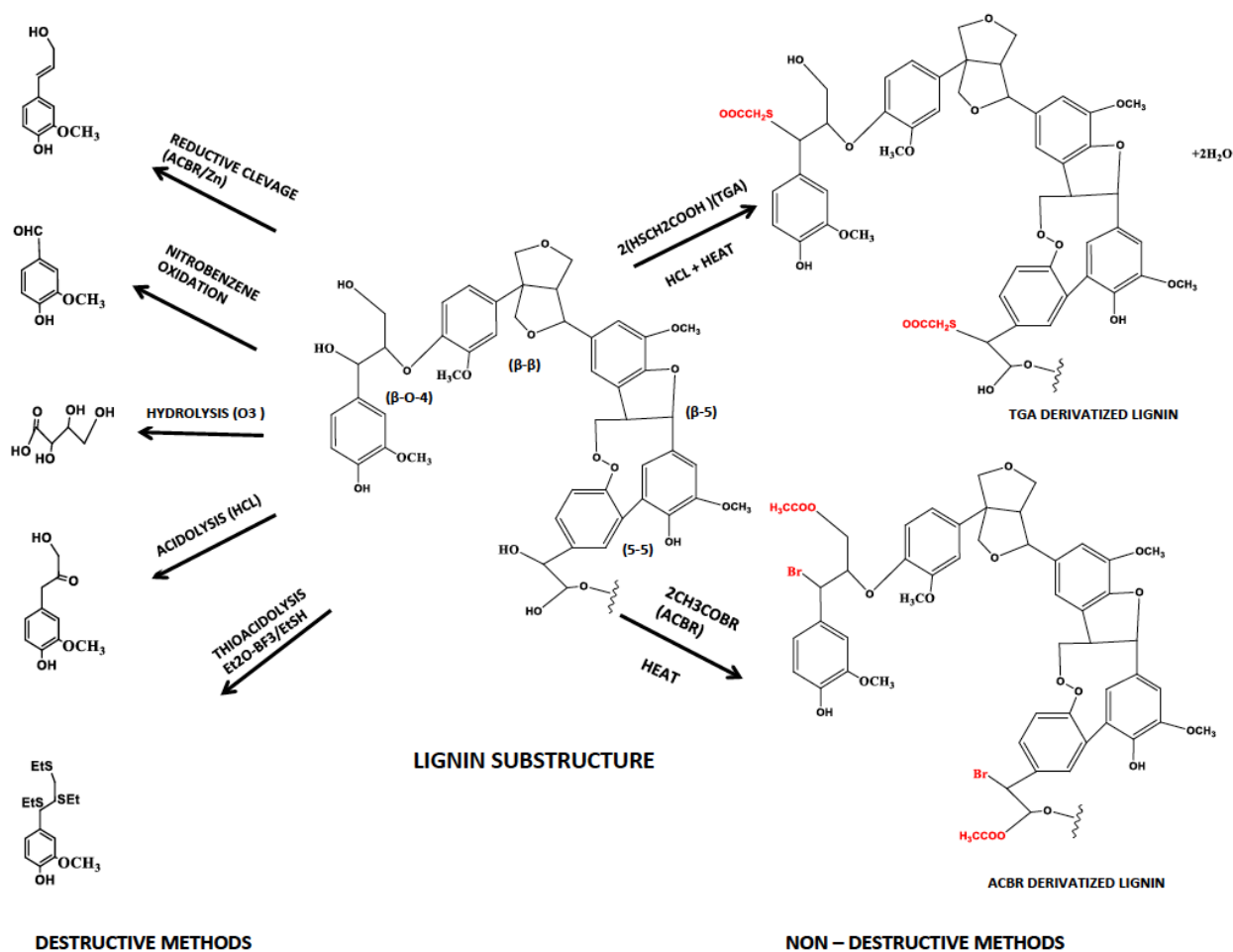


Figure 2. Lignin substructure and analytical methods. Destructive methods (acid hydrolysis, oxidation and reduction) result in breakdown products that can be used to quantify the lignin. Non-destructive methods like TGA (thioglycolic acid) and ACBR (acetyl bromide) add functional groups to the lignin structure, thereby modifying its solubility, which helps in quantifying the lignin content/composition using UV absorbance. (HCl- hydrochloric acid; BF₃- boron trifluoride; EtSH- ethanethiol; Et₂O- diethyl ether; Zn- zinc).

3.1.1. Degradative Analytical Techniques (Oxidation, Reduction, Hydrolysis and Acidolysis)

The oxidation of lignin is carried out using various oxidizing agents, like nitrobenzene, cupric oxide and potassium permanganate, which break down the side chains present in lignin, thereby leaving aromatic ring end products. For example, in nitrobenzene oxidation, lignin is reacted with nitrobenzene under high temperature in alkaline conditions resulting in oxidation products like guaiacol, vanillic acid and 5-carboxyvanillin [54]. This technique provides information on the aromatic ring structures present in lignin. Since oxidative techniques do not provide any information on the side chain structure present in lignin, ozonolysis, another degradative method, can be used to determine the side chain structure. In ozonolysis, the unsaturated chemical bonds present in the aromatic ring are targeted, leaving the side

chains in the form of carboxylic acid end products such as glycolic, oxalic and formic acids [55]. These two techniques complement each other in providing the detailed information on the lignin structure.

The most widely used degradative techniques to study the lignin subunit composition are acidolysis and thioacidolysis. In these methods, the most commonly present (70% of lignin) arylglycerol- β -arylether bond in lignin is broken, resulting in end products such as cinnamic acid, benzaldehyde, phenones and quinoids [56]. The thioacidolysis method has an advantage over acidolysis in detecting the 5-5' and 5-O-4' bonded dimers. However, both these methods have limitations in detecting compounds that contain only β -arylether bonds. Also, the use of highly acidic conditions makes it difficult to identify the end products obtained from these two methods [56].

In order to overcome the limitations in acidolysis and thioacidolysis methods, a much simpler method called as Derivatization Followed by Reductive Cleavage (DFRC) of lignin was developed. In this method, lignin is derivatized using acetyl bromide, and the resulting benzyl bromides are cleaved using zinc dust to produce cinnamyl alcohol derivatives [57]. By using the DFRC method, novel compounds like isochroman are identified in lignin. Though the presence of different chemical subunits in lignin is studied using different degradative techniques as stated earlier, the entire representation of different monomeric compounds present in lignin is usually undermined due to the selective targeting of chemical bonds by these different methods.

3.1.2. Non-Degradative Analytical Techniques (Thioglycolic Acid–TGA and Acetyl Bromide–ACBR)

In order to have an overall structural analysis of lignin and to study the macromolecular properties of lignin such as size, structure and degree of polymerization, non-degradative techniques like TGA and ACBR are used [58,59]. In these two methods, the lignin is derivatized by adding functional groups to the β -O'-4 units and then solubilizing the lignin in suitable solvents for further analysis. The solubilized lignin is analyzed by size exclusion chromatographic (SEC) techniques to study the size and conformation of the polymer. The native structure of polymer is preserved during both the extraction and derivatization procedures. (Figure 2 and Table 2).

Table 2. Lignin derivatization methods with targeted substrate & resulting products.

Derivatization Method	Targeted Substrate	Product	Reference
Nitrobenzene oxidation	Side chains of phenyl ring	Breaks side chains and leaves aromatic ring	[54]
Derivatization followed by Reductive Cleavage	α - & β -aryl ether bonds	4-acetoxy cinnamylacetate Coniferyl diacetate Sinapyl diacetate	[57]
Ozonolysis	Unsaturated bonds of phenyl ring	Carboxylic acid	[55]
Acidolysis	Aryl glycerol- β -aryl ether	Single ring aromatic compounds	[56]
Thio acidolysis	Aryl glycerol- β -aryl ether	Single ring aromatic compounds & dimers with 5-5 & 5-O-4 links	[56]
ThioGlycolic Acid	β -aryl ether bonds	Thioglycolic group substituted lignin polymer	[58]
Acetyl bromide	α - & β -aryl ether bonds	Acetyl bromide group substituted lignin polymer	[59]

However, the limitations of SEC are the approximate determination of the molar mass based on the UV absorbance and the lengthy elution time [60]. Moreover, the structure/conformation of the lignin polymer cannot be studied by the UV absorption method. To overcome this limitation, the dynamic light scattering method was developed to accurately determine the molar mass and shape distribution of the polymer [60]. The use of proper non-degradative extraction methods coupled with SEC can be used to determine the lignin structure.

4. Lignin Bioengineering for Efficient Utilization of Plant Biomass

For efficient utilization of plant biomass and reduction of pretreatment costs in bioethanol production, the recalcitrant matrix of cell wall polysaccharides has to be engineered to reduce the biomass recalcitrance. Lignin is considered as a major component that causes biomass recalcitrance. Reduction in lignin content by various methods resulted in improved biomass digestibility and reduced recalcitrance. Plant biomass with less lignin can be produced by down-regulating/knocking-out the lignin biosynthetic genes using various genetic and molecular techniques. On the other hand, improving lignin content is beneficial to increase the energy density of the biomass or to incorporate high value chemicals into the lignin polymer. Higher amounts of lignin or incorporation of a particular non-canonical monomer can be achieved by over-expressing lignin biosynthetic or phenol oxidizing genes in plants. The following section summarizes the recent research progress in lignin genetic engineering.

4.1. Reducing the Lignin Content/Altering the Composition for Easy Separation of Polysaccharides

As described in the lignin biosynthesis section, there are several enzymes and transporters involved in lignin polymer production. Both forward and reverse genetic studies helped in identification and characterization of the genes involved in the lignin biosynthetic pathway. Some of these studies resulted in immediate and direct practical applications. The oldest dated naturally occurring lignin mutants were first described in maize in 1924 [61] under the name of brown midrib mutants (*bmr*) due to the typical accumulation of a brown/red pigment in the midrib. The mutants were characterized by low lignin content and increased digestibility [62,63]. Screening of chemically induced mutant populations subsequently expanded the number of brown midrib lines in maize and sorghum [64]. At a later stage, the nature of the pigment was determined to be an intermediate of the lignin pathway, resulting from mutations in lignin biosynthetic genes, primarily the *COMT* gene [64]. In addition to *bmr* mutants, other forms of phenylpropanoid mutants were also described [65,66]. For instance, the sorghum *RG (REDforGREEN)* mutant displayed an antithetic leaf shoot lignin accumulation with an increase in the saccharification efficiency [65].

Gene expression manipulation of the lignin biosynthetic genes leads to altered lignin composition or content. Many studies using T-DNA insertion mutagenesis and RNA interference technologies showed the knock-out/knock-down of genes involved in lignin biosynthesis resulting in a considerable reduction (10%–50%) of overall lignin content of the transgenic plants [67]. The lignin reduction in plant biomass often comes with a fitness cost, which might compromise the plant's ability to withstand biotic and abiotic stresses. These genetically engineered low lignin plants are often weak and produce lower total biomass due to the lack of lignin deposition in structural cells like xylem, tracheary elements and vessels [67]. Additionally, genetic engineering of lignin can affect related secondary metabolite

biosynthesis (flavonoids, coumarins and phenolic compounds), influencing normal plant growth and development [67]. Hence, the lignin bioengineering must take these issues into account for designing the genetic engineering strategies.

Improved biomass digestibility, without reducing lignin content, can be achieved by altering the lignin composition. As described earlier, the lignin polymer is formed by free radical coupling of the available monomeric units in the apoplastic region. Enhancing the availability can result in higher incorporation of a particular monomer into the polymer. The manipulation of lignin composition results in altered properties. Over-expression of the ferulate 5-hydroxylase gene in *Arabidopsis* resulted in an increase in S subunit incorporation into the lignin [68]. The modified monomeric ratio may result in the altered properties of the lignin polymer due to the chemical properties of the monomers. The extent of methoxylation in the H, G and S subunits differs in the order $S > G > H$, and the extent of condensed lignin formation from these three monomers differs in the order $H > G > S$ [69]. Altered composition of these monomers has been shown to influence the biomass digestibility properties. Enhanced incorporation of G lignin negatively affected the glucose yield from the biomass when compared to the lignin enriched with S subunits in *Arabidopsis* cell walls [70]. This phenomenon was also observed in *COMT* down-regulated transgenic tobacco plants. Down-regulation of *COMT* led to an increased S/G ratio of the lignin and resulted in better digestibility, however, it was accompanied by a reduced lignin content [71]. Incorporation of alternative, non-canonical lignin monomers, like hydroxyl-benzaldehyde, resulted in a lower molecular weight lignin, leading to a better digestibility of *Arabidopsis* biomass without altering the lignin strength [72].

Apart from manipulating the lignin biosynthetic genes, lignin composition can be altered by manipulating the phenol oxidase genes. Since these enzymes have substrate specificity and reside in the apoplastic region to form free radicals for random polymerization, it is possible to manipulate the lignin composition with the help of these enzymes. Laccases have high substrate specificity and different substrates have been identified for different laccase enzymes [73]. Several studies indicated that the absence of a particular laccase caused reduction in total lignin content [74–76]. *LAC17* is specifically involved in the incorporation of G subunits into the lignin polymer [50], and simultaneous mutations in three laccase genes (*LAC4*, *LAC11* and *LAC17*) resulted in reduced lignin deposition, largely effecting the plant gross morphology [53].

The use of gene-and-tissue specific promoters, instead of constitutive promoters [cauliflower mosaic virus 35s (CaMV35s) or ubiquitin (UBI)], can address the potential for pleiotropic effects and minimize unintended detrimental effects of lignin bioengineering [77,78]. Tissue specific promoters in developing xylem and fibers have been identified in many plant species [79,80], thereby expanding the bio-engineering scenario. The organ/tissues/cell specificity can also be regulated using transcription factors (TF) [43]. In fact, TF have been used to reduce the lignin content in the supportive tissues like fibers, while maintaining the normal lignification in vessel elements [81]. New bioengineering methods like zinc finger nucleases (ZFN), transcription activation like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR) have been developed to edit the target genes by inducing point mutations in the genomes [82]. These methods employ endonucleases coupled with specific DNA binding elements that allow the editing of target genes. These bio-editing methods can be used to edit the lignin biosynthetic genes specifically expressed in supportive cells around the vessel cells, thereby creating mutant plants with reduced lignin content for better digestibility with less pleiotropic effects.

4.2. Enhancing Lignin Content to Produce High Energy Dense Feedstocks

Of all the plant cell wall polymers, lignin is highly reduced and energy dense with a high heating value [16,83]. The high lignin biomass can be used for the production of high-energy dense jet fuels, bioenergy and bioelectricity by thermochemical conversion methods [10,84]. Due to the cost involved in bioethanol production, the recent focus has been shifting towards the production of drop-in hydrocarbon biofuels and bioelectricity generation by thermal deconstruction processes such as pyrolysis and gasification respectively [85]. Hence, enhancing the lignin deposition will improve the feedstock suitability for the thermal conversion processes for the production of bioelectricity and biooil.

Alteration of a cell wall component (cellulose, lignin or hemicellulose) is often followed by a compensatory increase/decrease in other cell wall polymers to maintain the wall strength to withstand against the internal turgor pressure of the plant cells. Mutations in lignin biosynthetic genes result in an increase in non-cellulosic polysaccharide content [86] and ectopic lignification in cellulose mutants [87]. Ectopic lignin deposition in cells that otherwise have no lignin has been observed in various Arabidopsis cell wall mutants [88,89]. Increased deposition of ectopic lignin was observed in plants with reduced cellulose deposition, as seen with the *ectopic lignin 1 (eli 1)* mutant, which has a mutation in the *Cellulose Synthase 3* gene [87]. However, the increase in lignin was associated with an elevated defense response in *eli* mutants, resulting in reduced plant growth, as seen with many biotic and abiotic stress responses in plants [90]. This could result from the re-allocation of phenolic compounds for the biosynthesis of flavonoids that are involved in stress signaling [91]. These studies indicate an extensive signaling mechanism to maintain the cell wall strength and defense responses. Studies on Arabidopsis laccase T-DNA insertion mutants showed the existence of a feedback loop in controlling the expression of lignin biosynthetic genes [50]. The existence of signaling mechanisms that enable the maintenance of the wall strength by compensatory deposition of other cell wall polysaccharides is an attractive scenario. It will be important to identify these dedicated signaling mechanisms for an efficient bioengineering of the plant cell walls for enhanced lignin production and resistance.

Lignin content can be enhanced by over-expressing lignin biosynthetic genes or by down-regulating the negative regulators of lignin biosynthesis. The cotton (*Gossypium arboreum*) laccase (*GaLAC1*) gene, when over-expressed in poplar (*Populus spp.*) plants resulted in increased lignin content [92]. Also in poplar, the over-expression of a *Ferulate-5-hydroxylase* resulted in an increase of S subunits in the lignin polymer [93]. Several biotic and abiotic stresses influence the lignin biosynthesis in plants via TF [94], for example, in Arabidopsis *MYB58* and *MYB63I*, TF have been shown to activate lignin biosynthesis [95]. Similarly, microRNAs, negative regulators of gene expression, have been shown to regulate the lignin biosynthetic genes [96]. Apart from individual genes and TF, hormones have also been implicated in regulating the lignin biosynthesis. Overproduction of auxin, indole-3-acetic acid (IAA), resulted in an enhanced lignin content and an altered composition, indicating the role of hormones in the regulation of lignin biosynthesis [97]. Similarly, ethylene and gibberellins have also been implicated in lignin biosynthesis [98,99], indicating the possibility of altering the lignin content by external hormone applications.

Lignin composition can be altered to produce high value aromatic compounds. Since lignin polymerization occurs by random coupling of activated available monomers in the apoplasmic region, non-canonical lignin monomers, like epigallocatechin, can be incorporated into the lignin polymer [100].

Also, depending upon the breakdown end products needed, plant lignin can be altered to incorporate the compounds, thereby making plant biofactories for the production of valuable compounds like benzoate, cinnamate and tyramine.

5. Separation of Lignin from Other Polymers in Plant Biomass (Chemical and Biological)

Access to the individual cell wall polymers is a crucial step in determining the rate of conversion of the polymers into desired end products. The effective biofuel and biochemical production from plant biomass depends upon the biomass pretreatment, which depolymerizes the cell wall polymers, allowing easy access of enzymes to the polymers. Many methods have been developed in order to separate lignin from other polymers of plant biomass. Chemical and biological methods have been utilized with various rates of success depending on the desired end product from the separated lignin, the cost effective separation techniques and the amount of time devoted to the separation process. The following sections describe different chemical and biological separation processes.

5.1. Chemical-Based Lignin Separation

The study of natural lignin composition has been unsuccessful so far due to the inability to isolate and characterize lignin in its native form from plant biomass. Chemically breaking down lignin has helped the compositional study of this heteropolymer by targeting different bonds present in the lignin structure (Table 2), however, any isolation method or treatment of lignin alters its native structure/form [21,89]. Several methods have been developed to separate lignin from lignocellulosic biomass and further to depolymerize lignin into a usable form for production of different biofuels and bioproducts. These separation techniques produce technical lignins, which are classified according to the process applied: steam explosion, kraft, organosolv, alkaline oxidation, and pyrolysis lignins [101]. More knowledge on lignin composition and structure resulted from the analyses of technical lignins. Therefore, it is possible to speculate that new insights gained from studying the structure(s) and composition of the technical lignins will allow the development of more efficient methods to convert this lignin into useful biofuels and bioproducts.

The separation of lignin from hemicellulose and cellulose is known as fractionation. The most common lignin product of this fractionation process is called pulping liquor or kraft lignin, a liquid that results from the pulping process in paper manufacturing [102]. The pulping liquor is the main commercial source of lignin [6] and the lignin is extracted by precipitating it under low pH conditions in the presence strong minerals. Though this is the most simple of the fractionation processes, it causes colloid formation resulting in a low quality, impure lignin product [102]. Highly purified lignin of low molecular weight can be extracted more precisely using enzymes and other specific solvents which is an expensive process. Moreover, a recent technique using membranes for the separation of lignin by molecular weights is becoming more popular due to its higher specificity [102].

Lignin depolymerization is a process that breaks down the complex aromatic structures into simple compounds that can be used to make commercially important chemicals and biofuels. Chemically, lignin depolymerization can be accomplished by: (1) pyrolysis, (2) hydrogenolysis, (3) oxidation, (4) gasification and (5) combustion (Figure 3, [4]). The efficiency of degradation and the products produced by these methods, is dependent on the type of lignin and the method used to separate lignin

from cellulose and hemicellulose [21]. Pyrolysis is a complex process that involves thermal treatment of lignin in the absence of oxygen. The temperature treatment ranges from 300 °C to 600 °C. The absence of oxygen is necessary to prevent the reaction from further combusting into CO₂ [21]. The degree to which this process rearranges the ring structures of lignin is dependent upon the feedstock type, temperature, and heating rate [101].

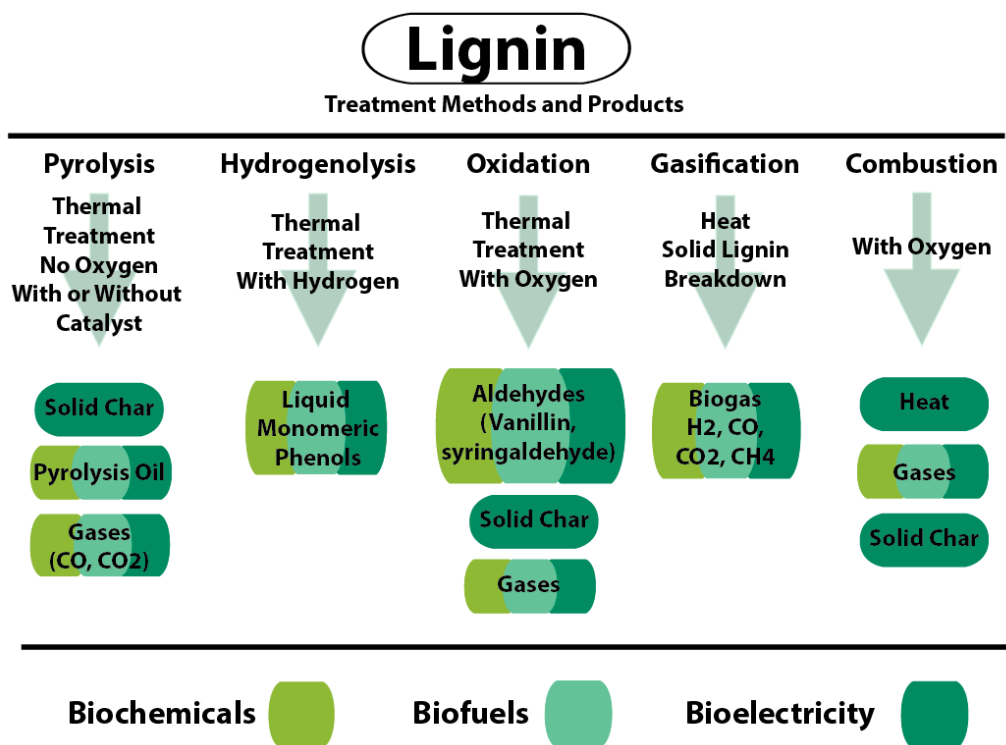


Figure 3. Chemical methods of lignin depolymerization and its end use in the production of chemicals, fuel and electricity. Lignin breakdown is carried out at high temperatures with or without added oxygen and hydrogen, depending upon the method. This results in varied levels of chemicals, liquid fuel and electricity produced. The color coding indicates the product type produced from the biomass processing.

The products of pyrolysis are small fragments of biomass material in either liquid form, called pyrolysis oil, or solid form, called solid char or biochar. Dehydrated sugars and organic acids make up the majority of the fragments produced. The difficulty with this method is converting these fragments to stable non-corrosive components [4]. Hydrogenolysis, also known as hydrocracking, is a type of pyrolysis with the addition of hydrogen. The hydrogen assists in the cleavage of bonds. This process creates more monomeric liquid phenols due to the lower reaction temperature [21]. Oxidation of lignin involves heating the lignin in the presence of oxygen. Lignin reacts well to this treatment due to the presence of many hydroxyl groups. Products from this treatment include aldehydes, gas, and solid char. Oxidants that can be used for this process include hydrogen peroxide, nitrobenzene, and metal oxides [21]. Gasification converts lignin into a gas called syngas consisting of CO₂, CO, CH₄, and H₂. It uses much higher temperatures than pyrolysis, between 700 °C and 1000 °C [101]. Once syngas is produced, cleaned and filtered to remove problematic chemical compounds, it can then be used to generate energy. One example of this process is given through a combined cycle turbine that uses gas and steam to produce

electricity [101]. Combustion occurs in the presence of oxygen at extremely high temperatures (around 800 °C to 1000 °C) and can be used to produce heat, electricity, gases and solid char [17,55].

5.2. Biological Degradation of Lignin

Biologically, lignin depolymerization from plant biomass can be achieved by a bioconversion method in which thermo-tolerant molds are used to delignify plant biomass [103]. Delignified substrate is then exposed to different bacterial and fungal strains to ferment the hexose and pentose sugars to produce bioethanol, which is then distilled out of the fermentation chamber [101]. Bioconversion is adapted from microbes, bacteria, fungi and termites that have been found to breakdown biomass for their survival [104–106]. Pilot studies on bioconversion of various biomass feedstocks using the fungus *Ceriporiopsis subvermispora* showed a glucose yield of around 25%–56%, where the yield increase was dependent upon the pretreatment time with the fungal cultures [106]. Fungal cellulolytic enzymes are commercially being used to breakdown non-lignin biomass, whereas, the applicability of laccases and peroxidases in lignin breakdown is still at the laboratory level [107]. There is increasing evidence that bacterial laccases and peroxidases, that are similar to their fungal counterparts, are involved in the degradation of lignin. Such bacterial laccases and peroxidases have been found in bacterial species grown in forest soils [108]. Identification of lignin degrading enzymes from *Thermus thermophilus* has also lead to the finding of novel laccases with an increased half-life at elevated temperatures [109]. In another study, the expression of the bacterial enzyme hydroxycinnamyl-CoA hydratase-lyase (HCHL) in *Arabidopsis* plants, produced hydroxybenzaldehyde derivatives of lignin monomers that improved saccharification of the biomass [72]. Apart from finding novel enzymes from microbial species, directed evolution can be used to improve the activity of enzymes currently produced by the known microbial species [110]. Microbial lignin degrading enzymes have multiple applications in food, pharmaceutical and fragrance industries. For instance, laccase mediated grafting of phenolics on cellulose has been used to produce packaging material with antimicrobial properties [111]. Moreover, the intermediate compounds of the lignin biosynthetic pathway, like hydroxycinnamic acids and aldehydes, possess antimicrobial and antifungal properties [6,112,113] as determined by their minimum killing concentration (MKC). This shows that microbial breakdown products from lignin of various origins could produce novel compounds with antimicrobial properties. Also, identification of novel microbial enzymes that can degrade phenolic compounds can be applied to the breakdown of aromatic pesticides [114] for bioremediation applications. Adoption of microbial enzymes to breakdown lignin in the pretreatment of biomass will improve the efficiency of lignin breakdown and reduce the use of chemicals that interfere in the saccharification process [115].

6. Applications of Lignin for Biofuel and Bioproducts

Lignin holds a great potential in different industries as a source for chemicals, fuels and other bioproducts. Though the calorific value of lignin is similar to ethanol (27 KJ/g), the high density of lignin makes this compound a potential source for bioenergy from high-lignin feedstocks [10,16]. Lignin is a common byproduct derived from different industries, including paper and pulp industries [21]. This industry alone creates 40–50 tons of lignin per year according to the International Lignin Institute, yet only 1.5% is currently used for purposes other than energy generation [102]. Lignin is also the main

waste product from bioethanol production. Some bioethanol producers dispose of this lignin as waste, while others utilize it to generate energy or for solid char production, an attempt to refill the carbon sink [6].

Besides its contribution to energy production, lignin is biodegradable, antimicrobial, an antioxidant, and CO₂ neutral [6,18,116]. Such diverse properties of lignin have accentuated the need to convert and use lignin in liquid fuel additives and commercially important chemicals [21]. Due to its diverse functional groups, lignin has the potential to be used as an absorbent agent, an emulsifier, a dispersant reagent in cement and gypsum blends, and as a chelating agent [117–120]. It has the potential to generate a wide range of chemicals and adhesives normally derived from fossil fuels [6,102]. Lignin is also being used for the production of controlled and slow release fertilizers, as well as in bioplastics [121]. Also, carbon fibers obtained from lignin are used to make high energy super capacitors as energy storage devices [122].

7. Conclusions

Lignin has the potential to serve as an alternative standalone fuel and can be a source for many valuable chemicals. Though there is considerable progress in the lignin biosynthetic pathway, the lignin composition and content could not be altered without any negative consequences. Many lignin biosynthetic mutants have reduced biomass and decreased resistance to pathogens [123]. However, by expressing novel microbial enzymes in plants, the nature of monomers involved in lignin polymerization can be altered, thereby reducing the molecular weight of lignin without compromising the biomass yield [72]. Key elemental research is needed for: (1) generating an altered lignin polymer without compromising the cell wall properties; (2) engineering lignin that can be easily deconstructed into specific bioproducts; (3) exploring novel enzymes that can be expressed to tailor the plant lignin composition and structure; (4) developing specialized methods for lignin isolation and controlled depolymerization for compositional analysis and industrial applications.

The bioproducts resulting from lignin can reduce our reliance on petroleum-based products, which is a necessity as oil reserves are not only limited but also non-renewable. Additionally, biological alteration of the lignin structure will reduce chemicals used for its breakdown in the bioethanol industry and thereby reduce the pretreatment costs and chemical waste.

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Conflict of interest

The authors declare no conflict of interest.

References

1. Alexandratos, N.; Bruinsma, J. *World Agriculture towards 2030/2050: The 2012 Revision*; ESA working paper No: 12-03; Food and Agriculture Organization of the United Nations (FAO): Rome, Italy, 2012.

2. Alexandratos, N. The Mediterranean diet in a world context. *Public Health Nutr.* **2006**, *9*, 111–117.
3. Perlack, R.D.; Wright, L.L.; Turhollow, A.F.; Graham, R.L.; Stokes, B.J.; Erbach, D.C. *Biomass as Feedstock for a Bioenergy and Bioproducts Industry: The Technical Feasibility of a Billion-Ton Annual Supply*; Technical Report; Oak Ridge National Laboratory (ORNL): Oak Ridge, TN, USA, 2005.
4. Sorek, N.; Yeats, T.H.; Szemenyei, H.; Youngs, H.; Somerville, C.R. The Implications of Lignocellulosic Biomass Chemical Composition for the Production of Advanced Biofuels. *BioScience* **2014**, *64*, doi:10.1093/biosci/bit037.
5. van Duren, I.; Voinov, A.; Arodudu, O.; Firrisa, M.T. Where to produce rapeseed biodiesel and why? Mapping European rapeseed energy efficiency. *Renew. Energy* **2015**, *74*, 49–59.
6. Doherty, W.O.S.; Mousavioun, P.; Fellows, C.M. Value-adding to cellulosic ethanol: Lignin polymers. *Industrial Crop. Prod.* **2011**, *33*, 259–276.
7. Searchinger, T.; Edwards, R.; Mulligan, D.; Heimlich, R.; Plevin, R. Do biofuel policies seek to cut emissions by cutting food? *Science* **2015**, *347*, 1420–1422.
8. Pimentel, D.; Burgess, M. Biofuel production using food. In *Environment, Development and Sustainability*; Springer: Berlin, Germany, 2014; Volume 16, pp. 1–3.
9. Galbe, M.; Zacchi, G. Pretreatment of lignocellulosic materials for efficient bioethanol production. *Adv. Biochem. Eng. Biotechnol.* **2007**, *108*, 41–65.
10. Mendu, V.; Shearin, T.; Campbell, J.E.; Stork, J.; Jae, J.; Crocker, M.; Huber, G.; DeBolt, S. Global bioenergy potential from high-lignin agricultural residue. *Proc. Natl. Acad. Sci.* **2012**, *109*, 4014–4019.
11. Stigka, E.K.; Paravantis, J.A.; Mihalakakou, G.K. Social acceptance of renewable energy sources: A review of contingent valuation applications. *Renew. Sustain. Energy Rev.* **2014**, *32*, 100–106.
12. Sticklen, M.B. Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. *Nat. Rev. Genet.* **2008**, *9*, 433–443.
13. Kajikawa, Y.; Takeda, Y. Structure of research on biomass and bio-fuels: A citation-based approach. *Technol. Forecast. Soc. Chang.* **2008**, *75*, 1349–1359.
14. Lark, T.J.; Salmon, J.M.; Gibbs, H.K. Cropland expansion outpaces agricultural and biofuel policies in the United States. *Environ. Res. Lett.* **2015**, *10*, 044003.
15. Qin, Z.; Zhuang, Q.; Zhu, X.; Cai, X.; Zhang, X. Carbon Consequences and Agricultural Implications of Growing Biofuel Crops on Marginal Agricultural Lands in China. *Environ. Sci. Technol.* **2011**, *45*, 10765–10772.
16. Mendu, V.; Harman-Ware, A.E.; Crocker, M.; Jae, J.; Stork, J.; Morton, S., 3rd; Placido, A.; Huber, G.; Debolt, S. Identification and thermochemical analysis of high-lignin feedstocks for biofuel and biochemical production. *Biotechnol. Biofuels* **2011**, *4*, doi:10.1186/1754-6834-4-43.
17. McKendry, P. Energy production from biomass (part 2): Conversion technologies. *Bioresour. Technol.* **2002**, *83*, 47–54.
18. Thakur, V.K.; Thakur, M.K. Recent advances in green hydrogels from lignin: a review. *Int. J. Biol. Macromol.* **2015**, *72*, 834–847.
19. Sluiter, J.B.; Ruiz, R.O.; Scarlata, C.J.; Sluiter, A.D.; Templeton, D.W. Compositional Analysis of Lignocellulosic Feedstocks. 1. Review and Description of Methods. *J. Agric. Food Chem.* **2010**, *58*, 9043–9053.

20. Lee, D.; Owens, V.N.; Boe, A.; Jeranyama, P. *Composition of Herbaceous Biomass Feedstocks*; North Central Sun Grant Center, South Dakota State University: Brookings, SD, USA, 2007; pp. 1–16.
21. Pandey, M.P.; Kim, C.S. Lignin Depolymerization and Conversion: A Review of Thermochemical Methods. *Chem. Eng. Technol.* **2011**, *34*, 29–41.
22. Klinke, H.B.; Thomsen, A.B.; Ahring, B.K. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Appl. Microbiol. Biotechnol.* **2004**, *66*, 10–26.
23. Xu, C.; Arancon, R.A.D.; Labidi, J.; Luque, R. Lignin depolymerisation strategies: towards valuable chemicals and fuels. *Chem. Soc. Rev.* **2014**, *43*, 7485–7500.
24. Nawrath, C.; Schreiber, L.; Franke, R.B.; Geldner, N.; Reina-Pinto, J.J.; Kunst, L. Apoplastic Diffusion Barriers in Arabidopsis. *Arab. Book* **2013**, *11*, doi:10.1199/tab.0167.
25. Boyce, C.K.; Zwieniecki, M.A.; Cody, G.D.; Jacobsen, C.; Wirick, S.; Knoll, A.H.; Holbrook, N.M. Evolution of xylem lignification and hydrogel transport regulation. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 17555–17558.
26. Bhuiyan, N.H.; Selvaraj, G.; Wei, Y.; King, J. Role of lignification in plant defense. *Plant Signal. Behav.* **2009**, *4*, 158–159.
27. Liljegren, S.J.; Ditta, G.S.; Eshed, Y.; Savidge, B.; Bowman, J.L.; Yanofsky, M.F. SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. *Nature* **2000**, *404*, 766–770.
28. Weng, J.K.; Chapple, C. The origin and evolution of lignin biosynthesis. *New Phytol.* **2010**, *187*, 273–285.
29. Uzal, E.N.; Gomez Ros, L.V.; Pomar, F.; Bernal, M.A.; Paradela, A.; Albar, J.P.; Ros Barcelo, A. The presence of sinapyl lignin in Ginkgo biloba cell cultures changes our views of the evolution of lignin biosynthesis. *Physiol. Plant.* **2009**, *135*, 196–213.
30. Boerjan, W.; Ralph, J.; Baucher, M. Lignin biosynthesis. *Annu. Rev. Plant Biol.* **2003**, *54*, 519–546.
31. Ragauskas, A.J.; Beckham, G.T.; Biddy, M.J.; Chandra, R.; Chen, F.; Davis, M.F.; Davison, B.H.; Dixon, R.A.; Gilna, P.; Keller, M.; *et al.* Lignin valorization: Improving lignin processing in the biorefinery. *Science* **2014**, *344*, doi:10.1126/science.1246843
32. Lewis, N.G.; Yamamoto, E. Lignin: Occurrence, biogenesis and biodegradation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1990**, *41*, 455–496.
33. Martone, P.T.; Estevez, J.M.; Lu, F.; Ruel, K.; Denny, M.W.; Somerville, C.; Ralph, J. Discovery of lignin in seaweed reveals convergent evolution of cell-wall architecture. *Curr. Biol.* **2009**, *19*, 169–175.
34. Woolhouse, H.W. *Advances in Botanical Research*; Academic Press: Waltham, MA, USA, 1981; Volume 8, p. 285.
35. Estevez, J.M.; Leonardi, P.I.; Alberghina, J.S. Cell wall carbohydrate epitopes in the green alga *Oedogonium bharuchae* F. minor (oedogoniales, chlorophyta). *J. Phycol.* **2008**, *44*, 1257–1268.
36. Kloareg, B.; Quatrano, R.S. Structure of the cell walls of marine algae and ecophysiological functions of the matrix polysaccharides. *Oceanogr. Mar. Biol. Annu. Rev.* **1988**, *26*, 259–315.

37. Wilson, M.A.; Sawyer, J.; Hatcher, P.G.; Lerch, H.E., III. 1,3,5-Hydroxybenzene structures in mosses. *Phytochemistry* **1989**, *28*, 1395–1400.
38. Siegel, S.M. Evidence for the Presence of Lignin in Moss Gametophytes. *Am. J. Bot.* **1969**, *56*, 175–179.
39. Ros, L.V.G.; Gabaldón, C.; Pomar, F.; Merino, F.; Pedreño, M.A.; Barceló, A.R. Structural motifs of syringyl peroxidases predate not only the gymnosperm–angiosperm divergence but also the radiation of tracheophytes. *New Phytol.* **2007**, *173*, 63–78.
40. Silva, G.B.; Ionashiro, M.; Carrara, T.B.; Crivellari, A.C.; Tiné, M.A.S.; Prado, J.; Carpita, N.C.; Buckeridge, M.S. Cell wall polysaccharides from fern leaves: Evidence for a mannan-rich Type III cell wall in *Adiantum raddianum*. *Phytochemistry* **2011**, *72*, 2352–2360.
41. Moozhiyil, M.; Pallauf, J. Chemical composition of the water fern, *salvinia molesta*, and its potential as feed source for ruminants. *Econ. Bot.* **1986**, *40*, 375–383.
42. Jouanin, L.; Lapierre, C. Lignins: Biosynthesis, Biodegradation and Bioengineering. In *Advances in Botanical Research*; Academic Press: Waltham, MA, USA, 2012; Volume 61, pp. 2–391.
43. Ralph, J.; Hatfield, R.D.; Quideau, S.; Helm, R.F.; Grabber, J.H.; Jung, H.-J.G. Pathway of *p*-Coumaric Acid Incorporation into Maize Lignin as Revealed by NMR. *J. Am. Chem. Soc.* **1994**, *116*, 9448–9456.
44. Saulnier, L.; Crépeau, M.-J.; Lahaye, M.; Thibault, J.-F.; Garcia-Conesa, M.T.; Kroon, P.A.; Williamson, G. Isolation and structural determination of two 5,5'-diferuloyl oligosaccharides indicate that maize heteroxylans are covalently cross-linked by oxidatively coupled ferulates. *Carbohydr. Res.* **1999**, *320*, 82–92.
45. Vogel, J. Unique aspects of the grass cell wall. *Curr. Opin. Plant Biol.* **2008**, *11*, 301–307.
46. Alejandro, S.; Lee, Y.; Tohge, T.; Sudre, D.; Osorio, S.; Park, J.; Bovet, L.; Lee, Y.; Geldner, N.; Fernie, A.R.; *et al.* AtABCG29 is a monolignol transporter involved in lignin biosynthesis. *Curr. Biol.* **2012**, *22*, 1207–1212.
47. Eudes, A.; Liang, Y.; Mitra, P.; Loque, D. Lignin bioengineering. *Curr. Opin. Biotechnol.* **2014**, *26*, 189–198.
48. Terashima, N.; Yoshida, M.; Hafrén, J.; Fukushima, K.; Westermark, U. Proposed supramolecular structure of lignin in softwood tracheid compound middle lamella regions. In *Holzforschung*; Walter de Gruyter: Berlin, Germany, 2012; Volume 66, pp. 907–915.
49. Giardina, P.; Sannia, G. Laccases: Old enzymes with a promising future. *Cell. Mol. Life Sci.* **2015**, *72*, 855–856.
50. Berthet, S.; Demont-Caulet, N.; Pollet, B.; Bidzinski, P.; Cezard, L.; Le Bris, P.; Borrega, N.; Herve, J.; Blondet, E.; Balzergue, S.; *et al.* Disruption of LACCASE4 and 17 results in tissue-specific alterations to lignification of *Arabidopsis thaliana* stems. *Plant. Cell.* **2011**, *23*, 1124–1137.
51. Schuetz, M.; Benske, A.; Smith, R.A.; Watanabe, Y.; Tobimatsu, Y.; Ralph, J.; Demura, T.; Ellis, B.; Samuels, A.L. Laccases direct lignification in the discrete secondary cell wall domains of protoxylem. *Plant Physiol.* **2014**, *166*, 798–807.
52. Wang, Y.; Bouchabké-Coussa, O.; Le Bris, P.; Antelme, S.; Soulhat, C.; Gineau, E.; Dalmais, M.; Bendahmane, A.; Morin, H.; Mouille, G.; *et al.* LACCASE 5 is required for lignification of the *Brachypodium distachyon* culm. *Plant Physiol.* **2015**, *168*, 192–204.

53. Zhao, Q.; Nakashima, J.; Chen, F.; Yin, Y.; Fu, C.; Yun, J.; Shao, H.; Wang, X.; Wang, Z.-Y.; Dixon, R.A. LACCASE Is Necessary and Nonredundant with PEROXIDASE for Lignin Polymerization during Vascular Development in Arabidopsis. *Plant Cell* **2013**, *25*, 3976–3987.
54. Chen, C.L. Nitrobenzene and Cupric Oxide Oxidations. In *Methods in Lignin Chemistry*; Lin, S., Dence, C., Eds.; Springer: Berlin/Heidelberg, Germany, 1992; pp. 301–321.
55. Sarkanen, K.V.; Islam, A.; Anderson, C.D. Ozonation. In *Methods in Lignin Chemistry*, Lin, S., Dence, C., Eds.; Springer: Berlin/Heidelberg, Germany, 1992; pp. 387–406.
56. Lundquist, K.; Kirk, T.K. Acid degradation of lignin. IV. Analysis of lignin acidolysis products by gas chromatography, using trimethylsilyl derivatives. *Acta Chem. Scand.* **1971**, *25*, 889–894.
57. Fachuang, L.; John, R. Efficient Ether Cleavage in Lignins: The Derivatization Followed by Reductive Cleavage Procedure as a Basis for New Analytical Methods. In *Lignin and Lignan Biosynthesis*; American Chemical Society: Washington, DC, USA, 1998; Volume 697, pp. 294–322.
58. Bruce, R.J.; West, C.A. Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. *Plant Physiol.* **1989**, *91*, 889–897.
59. Morrison, I.M. Improvements in the acetyl bromide technique to determine lignin and digestibility and its application to legumes. *J. Sci. Food Agric.* **1972**, *23*, 1463–1469.
60. Gidh, A.V.; Decker, S.R.; Vinzant, T.B.; Himmel, M.E.; Williford, C. Determination of lignin by size exclusion chromatography using multi angle laser light scattering. *J. Chromatogr. A* **2006**, *1114*, 102–110.
61. Jorgenson, L.R. Brown Midrib in Maize and its Linkage Relations. *Agron. J.* **1931**, *23*, 549–557.
62. Kuc, J.; Nelson, O.E. The abnormal lignins produced by the brown-midrib mutants of maize: I. The brown-midrib-1 mutant. *Arch. Biochem. Biophys.* **1964**, *105*, 103–113.
63. Kuc, J.; Nelson, O.E.; Flanagan, P. Degradation of abnormal lignins in the brown-midrib mutants and double mutants of maize. *Phytochemistry* **1968**, *7*, 1435–1436.
64. Saballos, A.; Vermerris, W.; Rivera, L.; Ejeta, G. Allelic Association, Chemical Characterization and Saccharification Properties of brown midrib Mutants of Sorghum (*Sorghum bicolor* (L.) Moench). *Bioenergy Res.* **2008**, *1*, 193–204.
65. Petti, C.; Harman-Ware, A.; Tateno, M.; Kushwaha, R.; Shearer, A.; Downie, A.; Crocker, M.; DeBolt, S. Sorghum mutant RG displays antithetic leaf shoot lignin accumulation resulting in improved stem saccharification properties. *Biotechnol. Biofuels* **2013**, *6*, doi:10.1186/1754-6834-6-146.
66. Mir Derikvand, M.; Sierra, J.; Ruel, K.; Pollet, B.; Do, C.-T.; Thévenin, J.; Buffard, D.; Jouanin, L.; Lapierre, C. Redirection of the phenylpropanoid pathway to feruloyl malate in Arabidopsis mutants deficient for cinnamoyl-CoA reductase 1. *Planta* **2008**, *227*, 943–956.
67. Poovaiah, C.R.; Nageswara-Rao, M.; Soneji, J.R.; Baxter, H.L.; Stewart, C.N., Jr. Altered lignin biosynthesis using biotechnology to improve lignocellulosic biofuel feedstocks. *Plant Biotechnol. J.* **2014**, *12*, 1163–1173.
68. Franke, R.; McMichael, C.M.; Meyer, K.; Shirley, A.M.; Cusumano, J.C.; Chapple, C. Modified lignin in tobacco and poplar plants over-expressing the Arabidopsis gene encoding ferulate 5-hydroxylase. *Plant J.* **2000**, *22*, 223–234.
69. Sangha, A.K.; Parks, J.M.; Standaert, R.F.; Ziebell, A.; Davis, M.; Smith, J.C. Radical Coupling Reactions in Lignin Synthesis: A Density Functional Theory Study. *J. Phys. Chem. B* **2012**, *116*, 4760–4768.

70. Li, X.; Ximenes, E.; Kim, Y.; Slininger, M.; Meilan, R.; Ladisch, M.; Chapple, C. Lignin monomer composition affects Arabidopsis cell-wall degradability after liquid hot water pretreatment. *Biotechnol. Biofuels* **2010**, *3*, doi:10.1186/1754-6834-3-27.
71. Sewalt, V.; Ni, W.; Blount, J.W.; Jung, H.G.; Masoud, S.A.; Howles, P.A.; Lamb, C.; Dixon, R.A. Reduced Lignin Content and Altered Lignin Composition in Transgenic Tobacco Down-Regulated in Expression of L-Phenylalanine Ammonia-Lyase or Cinnamate 4-Hydroxylase. *Plant Physiol.* **1997**, *115*, 41–50.
72. Eudes, A.; George, A.; Mukerjee, P.; Kim, J.S.; Pollet, B.; Benke, P.I.; Yang, F.; Mitra, P.; Sun, L.; Cetinkol, O.P.; *et al.* Biosynthesis and incorporation of side-chain-truncated lignin monomers to reduce lignin polymerization and enhance saccharification. *Plant Biotechnol. J.* **2012**, *10*, 609–620.
73. Reiss, R.; Ihssen, J.; Richter, M.; Eichhorn, E.; Schilling, B.; Thöny-Meyer, L. Laccase versus Laccase-Like Multi-Copper Oxidase: A Comparative Study of Similar Enzymes with Diverse Substrate Spectra. *PLoS ONE* **2013**, *8*, doi:10.1371/journal.pone.0065633.
74. Cai, X.; Davis, E.J.; Ballif, J.; Liang, M.; Bushman, E.; Haroldsen, V.; Torabinejad, J.; Wu, Y. Mutant identification and characterization of the laccase gene family in Arabidopsis. *J. Exp. Bot.* **2006**, *57*, 2563–2569.
75. Pourcel, L.; Routaboul, J.-M.; Kerhoas, L.; Caboche, M.; Lepiniec, L.Ø.; Debeaujon, I. TRANSPARENT TESTA10 Encodes a Laccase-Like Enzyme Involved in Oxidative Polymerization of Flavonoids in Arabidopsis Seed Coat. *Plant Cell* **2005**, *17*, 2966–2980.
76. Liang, M.; Davis, E.; Gardner, D.; Cai, X.; Wu, Y. Involvement of *AtLAC15* in lignin synthesis in seeds and in root elongation of Arabidopsis. *Planta* **2006**, *224*, 1185–1196.
77. Bhinu, V.S.; Li, R.; Huang, J.; Kaminskyj, S.; Sharpe, A.; Hannoufa, A. Perturbation of lignin biosynthesis pathway in Brassica napus (canola) plants using RNAi. *Can. J. Plant Sci.* **2009**, *89*, 441–453.
78. Guo, D.; Chen, F.; Inoue, K.; Blount, J.W.; Dixon, R.A. Downregulation of caffeic acid 3-O-methyltransferase and caffeoyl CoA 3-O-methyltransferase in transgenic alfalfa. impacts on lignin structure and implications for the biosynthesis of G and S lignin. *Plant Cell* **2001**, *13*, 73–88.
79. Ko, J.H.; Kim, H.T.; Hwang, I.; Han, K.H. Tissue-type-specific transcriptome analysis identifies developing xylem-specific promoters in poplar. *Plant Biotechnol. J.* **2012**, *10*, 587–596.
80. Sakamoto, S.; Mitsuda, N. Reconstitution of a secondary cell wall in a secondary cell wall-deficient Arabidopsis mutant. *Plant Cell Physiol.* **2015**, *56*, 299–310.
81. Yang, F.; Mitra, P.; Zhang, L.; Prak, L.; Verhertbruggen, Y.; Kim, J.S.; Sun, L.; Zheng, K.; Tang, K.; Auer, M.; *et al.* Engineering secondary cell wall deposition in plants. *Plant Biotechnol. J.* **2013**, *11*, 325–335.
82. Gaj, T.; Gersbach, C.A.; Barbas, C.F., 3rd. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* **2013**, *31*, 397–405.
83. White, R.H. *Effect of Lignin Content and Extractives on the Higher Heating Value of Wood*; Society of Wood Science and Technology: Madison, WI, USA, 1987; Volume 19, pp. 446–452.
84. Marsh, G. Biofuels: Aviation alternative? *Renew. Energy Focus* **2008**, *9*, 48–51.
85. Regalbuto, J.R. Cellulosic Biofuels—Got Gasoline? *Science* **2009**, *325*, 822–824.

86. Van Acker, R.; Vanholme, R.; Storme, V.; Mortimer, J.C.; Dupree, P.; Boerjan, W. Lignin biosynthesis perturbations affect secondary cell wall composition and saccharification yield in *Arabidopsis thaliana*. *Biotechnol. Biofuels* **2013**, *6*, 46.
87. Cano-Delgado, A.; Penfield, S.; Smith, C.; Catley, M.; Bevan, M. Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. *Plant J.* **2003**, *34*, 351–362.
88. Rogers, L.A.; Dubos, C.; Surman, C.; Willment, J.; Cullis, I.F.; Mansfield, S.D.; Campbell, M.M. Comparison of lignin deposition in three ectopic lignification mutants. *New Phytol.* **2005**, *168*, 123–140.
89. Hématy, K.; Sado, P.-E.; Van Tuinen, A.; Rochange, S.; Desnos, T.; Balzergue, S.; Pelletier, S.; Renou, J.-P.; Höfte, H. A Receptor-like Kinase Mediates the Response of *Arabidopsis* Cells to the Inhibition of Cellulose Synthesis. *Curr. Biol.* **2007**, *17*, 922–931.
90. Moura, J.C.; Bonine, C.A.; de Oliveira Fernandes Viana, J.; Dornelas, M.C.; Mazzafera, P. Abiotic and biotic stresses and changes in the lignin content and composition in plants. *J. Integr. Plant Biol.* **2010**, *52*, 360–376.
91. Besseau, S.; Hoffmann, L.; Geoffroy, P.; Lapierre, C.; Pollet, B.; Legrand, M. Flavonoid accumulation in *Arabidopsis* repressed in lignin synthesis affects auxin transport and plant growth. *Plant Cell* **2007**, *19*, 148–162.
92. Wang, J.; Zhu, M.L.; Wei, Z.M. Cotton laccase gene overexpression in transgenic *Populus alba* var. *pyramidalis* and its effects on the lignin biosynthesis in transgenic plants. *J. Mol. Cell Biol.* **2008**, *41*, 11–18. (In Chinese)
93. Stewart, J.J.; Akiyama, T.; Chapple, C.; Ralph, J.; Mansfield, S.D. The effects on lignin structure of overexpression of ferulate 5-hydroxylase in hybrid poplar. *Plant Physiol.* **2009**, *150*, 621–635.
94. Zhao, Q.; Dixon, R.A. Transcriptional networks for lignin biosynthesis: More complex than we thought? *Trends Plant Sci.* **2011**, *16*, 227–233.
95. Zhou, J.; Lee, C.; Zhong, R.; Ye, Z.-H. MYB58 and MYB63 Are Transcriptional Activators of the Lignin Biosynthetic Pathway during Secondary Cell Wall Formation in *Arabidopsis*. *Plant Cell* **2009**, *21*, 248–266.
96. Ong, S.S.; Wickneswari, R. Characterization of microRNAs expressed during secondary wall biosynthesis in *Acacia mangium*. *PLoS ONE* **2012**, *7*, doi:10.1371/journal.pone.0049662.
97. Sitbon, F.; Hennion, S.; Little, C.H.A.; Sundberg, B. Enhanced ethylene production and peroxidase activity in IAA-overproducing transgenic tobacco plants is associated with increased lignin content and altered lignin composition. *Plant Sci.* **1999**, *141*, 165–173.
98. Rhodes, M.J.C.; Woollorton, L.S.C. Stimulation of phenolic acid and lignin biosynthesis in swede root tissue by ethylene. *Phytochemistry* **1973**, *12*, 107–118.
99. Peng, D.; Chen, X.; Yin, Y.; Lu, K.; Yang, W.; Tang, Y.; Wang, Z. Lodging resistance of winter wheat (*Triticum aestivum* L.): Lignin accumulation and its related enzymes activities due to the application of paclobutrazol or gibberellin acid. *Field Crops Res.* **2014**, *157*, 1–7.
100. Elumalai, S.; Tobimatsu, Y.; Grabber, J.H.; Pan, X.; Ralph, J. Epigallocatechin gallate incorporation into lignin enhances the alkaline delignification and enzymatic saccharification of cell walls. *Biotechnol. Biofuels* **2012**, *5*, doi:10.1186/1754-6834-5-59.
101. Wang, H.; Tucker, M.; Ji, Y. Recent Development in Chemical Depolymerization of Lignin: A Review. *J. Appl. Chem.* **2013**, *2013*, doi:10.1155/2013/838645.

102. Toledano, A.; García, A.; Mondragon, I.; Labidi, J. Lignin separation and fractionation by ultrafiltration. *Sep. Purif. Technol.* **2010**, *71*, 38–43.
103. López-Abelairas, M.; Álvarez Pallín, M.; Salvachúa, D.; Lú-Chau, T.; Martínez, M.J.; Lema, J.M. Optimisation of the biological pretreatment of wheat straw with white-rot fungi for ethanol production. *Bioprocess Biosyst. Eng.* **2013**, *36*, 1251–1260.
104. Rubin, E.M. Genomics of cellulosic biofuels. *Nature* **2008**, *454*, 841–845.
105. Sanchez, C. Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnol. Adv.* **2009**, *27*, 185–194.
106. Wan, C.; Li, Y. Effectiveness of microbial pretreatment by *Ceriporiopsis subvermispora* on different biomass feedstocks. *Bioresour. Technol.* **2011**, *102*, 7507–7512.
107. Dhiman, S.S.; Haw, J.-R.; Kalyani, D.; Kalia, V.C.; Kang, Y.C.; Lee, J.-K. Simultaneous pretreatment and saccharification: Green technology for enhanced sugar yields from biomass using a fungal consortium. *Bioresour. Technol.* **2015**, *179*, 50–57.
108. Kellner, H.; Luis, P.; Zimdars, B.; Kiesel, B.; Buscot, F. Diversity of bacterial laccase-like multicopper oxidase genes in forest and grassland Cambisol soil samples. *Soil Biol. Biochem.* **2008**, *40*, 638–648.
109. Miyazaki, T.; Miyazaki, J.; Yamane, H.; Nishiyama, M. alpha-Aminoadipate aminotransferase from an extremely thermophilic bacterium, *Thermus thermophilus*. *Microbiology* **2004**, *150*, 2327–2334.
110. Johannes, T.W.; Zhao, H. Directed evolution of enzymes and biosynthetic pathways. *Curr. Opin. Microbiol.* **2006**, *9*, 261–267.
111. Elegir, G.; Kindl, A.; Sadocco, P.; Orlandi, M. Development of antimicrobial cellulose packaging through laccase-mediated grafting of phenolic compounds. *Enzyme Microb. Technol.* **2008**, *43*, 84–92.
112. Baurhoo, B.; Ruiz-Feria, C.A.; Zhao, X. Purified lignin: Nutritional and health impacts on farm animals—A review. *Anim. Feed Sci. Technol.* **2008**, *144*, 175–184.
113. Barber, M.S.; McConnell, V.S.; DeCaux, B.S. Antimicrobial intermediates of the general phenylpropanoid and lignin specific pathways. *Phytochemistry* **2000**, *54*, 53–56.
114. Haggblom, M.M. Microbial breakdown of halogenated aromatic pesticides and related compounds. *FEMS Microbiol. Rev.* **1992**, *9*, 29–71.
115. Luo, C.; Brink, D.L.; Blanch, H.W. Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol. *Biomass Bioenergy* **2002**, *22*, 125–138.
116. Laurichesse, S.; Avérous, L. Chemical modification of lignins: Towards biobased polymers. *Prog. Polym. Sci.* **2014**, *39*, 1266–1290.
117. Yang, D.; Qiu, X.; Zhou, M.; Lou, H. Properties of sodium lignosulfonate as dispersant of coal water slurry. *Energy Convers. Manag.* **2007**, *48*, 2433–2438.
118. Boeriu, C.G.; Bravo, D.; Gosselink, R.J.A.; van Dam, J.E.G. Characterisation of structure-dependent functional properties of lignin with infrared spectroscopy. *Ind. Crops Prod.* **2004**, *20*, 205–218.
119. Sena-Martins, G.; Almeida-Vara, E.; Duarte, J.C. Eco-friendly new products from enzymatically modified industrial lignins. *Ind. Crops Prod.* **2008**, *27*, 189–195.

120. Mohan, D.; Pittman, C.U., Jr.; Steele, P.H. Single, binary and multi-component adsorption of copper and cadmium from aqueous solutions on Kraft lignin—A biosorbent. *J. Colloid Interface Sci.* **2006**, *297*, 489–504.
121. Norgren, M.; Edlund, H. Lignin: Recent advances and emerging applications. *Curr. Opin. Colloid Interface Sci.* **2014**, *19*, 409–416.
122. Hu, S.; Zhang, S.; Pan, N.; Hsieh, Y.-L. High energy density supercapacitors from lignin derived submicron activated carbon fibers in aqueous electrolytes. *J. Power Sources* **2014**, *270*, 106–112.
123. Pedersen, J.F.; Vogel, K.P.; Funnell, D.L. Impact of Reduced Lignin on Plant Fitness. *Crop. Sci.* **2005**, *45*, 812–819.

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