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Bioethanol: Feedstock Alternatives, Pretreatments, Lignin Chemistry, and the Potential for Green Value-Added Lignin Co-Products

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Abstract

Major challenges still exist in the cost-competitive production of cellulosic ethanol. Lignocellulose deconstruction using physiochemical pretreatments continues to be the most efficient way to remove lignin and expose cellulose for enzyme-mediated sugar reduction. Recent interest in the development of "value-added" lignin co-products and the desire to reduce the use of hazardous materials has prompted a resurgence of interest in alternative lignocellulosic feedstock and research into environment-friendly biological pretreatments. This article provides an overview of bioethanol processes and economics, standard and alternative feedstocks, physiochemical and biological pretreatments, and lignin chemistry. The chemistry and mode of action of ligninolytic and cellulolytic enzymes naturally expressed by white-rot and brown-rot fungi are described. This comprehensive review offers a renewed perspective on alternative high-lignin content cellulosic feedstock - specifically, bamboo - and the potential for microbial-based pretreatments that release cellulose for enzymatic breakdown and the subsequent fermentation of reduced sugars, while leaving lignin structures intact for conversion to valuable co-products.

Keywords: Bioethanol; Polysaccharides; Cellulase; Lignocellulosic

Introduction

Bioethanol has long been acknowledged as an environment-friendly, low-cost alternative to fossil fuel. However, it was not until the 1970s that lignocellulosic feedstocks (as opposed to corn or sugarcane) were considered for the production of bioethanol [1]. Since then, significant research has been conducted to optimize the process and reduce costs associated with cellulosic ethanol production. Still, major challenges must be overcome before lignocellulosic bioethanol can be produced as efficiently as corn-based and sugarcane-based ethanol. A major bottleneck in the cost-efficient production of cellulosic ethanol is the pretreatment stage of biomass deconstruction. Current pretreatment methods are largely limited to physical and chemical pretreatments, such as shredding, milling, grinding, pulverizing, acid digestion, alkaline digestion, ammonia fiber explosion, steam explosion, and combinations of these processes. Most of these physio-chemical pretreatments are efficient at deconstructing lignocellulose, but they often require high-energy inputs and the disposal of hazardous wastes, thereby increasing overall production costs [2,3].

The goal of any pretreatment is to increase enzyme access to targeted polysaccharides (i.e., cellulose) and most act to breakdown and remove lignin from the lignocellulosic matrix to achieve this goal. More recently, abiotic (enzyme-mediated) and biotic (microbial) pretreatments have been employed as environment-friendly alternatives to chemical-based pretreatments. Abiotic pretreatments usually require the use of costly enzymes to cleave key bonds between lignin and polysachharides or within the holocellulose itself. Although some progress has been made in the development of enzyme cocktails [4,5] and in the design of scaffold-bound multi-enzyme systems [6-9], current enzyme pretreatments for lignocellulosic biomass are still too costly for cellulosic ethanol to effectively compete with molasses/cane juice (i.e., sugarcane-based) and starch (i.e., corn-based) ethanol.

Microbial pretreatments include the use of active cultures (i.e., bacterial and/or fungal) to begin the lignocellulose deconstruction process [10-14]. The potential use of fungi as principal agents in biological pretreatments for a variety of feedstocks is appealing since it is from select fungal species that some of the most active cellulases and lignolytic enzymes have been discovered. However, for bioethanol production, attempts at fungal-based biological pretreatments have not proven economically viable, primarily due to the amount of time required for the fungi to adequately breakdown bulk biomass. Chemical pretreatments are orders of magnitude faster at removing lignin and disrupting the matrix to increase surface area thus improving cellulase access to cellulose. Despite this drawback, with proper scheduling and materials handling, microbial pretreatments can be effective for the environmentally conscientious producer. Interestingly, current literature on the potential use of fungi for degrading lignocellulose focuses almost exclusively on white-rot fungi and the soft-rot fungus, *Trichoderma reesei* [4,15-18]. However, recent reports from the microbiology community suggest that brown-rot fungi should not be discounted by the biotechnology sector since valuable lignin co-products may be generated as part of the delignification process [13,19,20].

In this paper, we provide an overview of biofuel feedstocks, pretreatments, and molecular targets in the lignocellulosic matrix with focus on lignin and the disruption of bonds between lignin and carbohydrates. This includes a discussion of lignin extraction methods and the economics of valuable lignin co-products. We then focus on specific physio-chemical and biological pretreatment options with emphasis on the potential development of microbial-based lignocellulose deconstruction strategies, particularly those that employ fungi. This includes details of lignocellulose breakdown

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via the action of enzyme systems and low-molecular weight compounds. Lastly, we revisit the potential value of alternative feedstocks - specifically bamboo - in the production of second-generation bioethanol, in light of new advances in lignin co-products development and recent discoveries in brown-rot fungal decay chemistry.

First Generation Biofuels (FGBs)

The current ethanol market is largely composed of corn-based ethanol produced in the US and Europe and sugarcane-based ethanol produced by countries such as Brazil and India [21]. Both of these feedstocks result in the production of what is known as first-generation biofuel (FGBs). These may be considered *renewable* alternatives to fossil fuels but whether they are *sustainable* is debated. Since FGB feedstocks use resources that could be used to grow food for human and livestock consumption (e.g., land and water), it is argued that these biofuels directly compete with food supplies and result in a *food-versus-fuel dilemma* thus rendering them unsustainable over the long-term.

In terms of process bottlenecks, corn-based ethanol production is considered efficient and has been extensively developed. Corn ethanol is produced by grinding corn kernel to access starch, a polysaccharide accounting for ~73% of the total kernel weight. The starch is then enzymatically hydrolyzed into monomeric glucose by α-amylases without any major pretreatment steps. The resulting glucose is fermented into ethanol and CO_2 by the yeast *Saccharomyces cerevisiae* or other engineered microorganisms [22-24].

Sugarcane-based ethanol is derived primarily from sugarcane juice and/or the molasses left over after the cane juice has been extracted for conversion into culinary sugar. The production of sugarcane ethanol is even simpler than corn ethanol since the primary substrate, sucrose, can be directly fermented into ethanol by industrial yeasts without need for additional enzymatic pretreatments [25].

Second Generation Biofuels (SGBs)

Second-generation biofuels (SGBs) are cellulosic ethanol products, which utilize cellulose, the single most abundant polymer on Earth, as the primary substrate. Breaking down cellulose into cellobiose and then into glucose monomers for fermentation can be effectively accomplished given a pure cellulose substrate. However, it has proven very challenging to develop a simple cost-competitive way to deconstruct the lignocellulosic matrix within which the cellulose is embedded in common SGB feedstocks such as corn stover, bagasse, wheat straw and grasses. These feedstocks typically have very little commercial value and are often left in the fields, burned, or at best are converted into animal feed. Arguably, the development of efficient and environment-friendly, lignocellulosic biomass deconstruction technologies would permit the production of ethanol without compromising the global food supply [26].

Unlike FGBs, SGB feedstocks requires significant pretreatment to breakdown the lignocellulosic matrix of the plant cell walls in order to increase enzyme access to the target polysaccharides - cellulose and hemicellulose - which are then converted into simpler sugars via enzyme-mediated reduction reactions [10,26-29]. Depending upon type of pretreatment, bonds between lignin and holocellulose may be targeted and/or bonds within the lignin structure may be targeted. Subsequent enzymatic treatments will target holocellulose itself to reduce these complex polysaccharides to fermentable sugars.

Feedstock selection for second generation biofuels

Selection of feedstocks for SGB production depends upon multiple factors including: potential yield per acre (or km^2); the agricultural inputs required, such as fertilizers and water; the proximity of feedstock producers to ethanol production facilities; and other biomass characteristics. Since the majority of ethanol plants are clustered in the US, Europe, Brazil, China and India which are the primary producers of corn, wheat, sugarcane, and rice, respectively [30], exploration of lignocellulosic feedstock options has largely focused on inedible or "waste" components from these high-volume crops (e.g., stover, straw, bagasse). Other potential cellulosic feedstocks have also been considered including hardwoods, softwoods, and grasses. However, these and other feedstock types are often understudied or overlooked because of high lignin content. Interestingly, some of these alternative feedstocks can take advantage of nutrient-poor or steeply-sloping soils, which are not suitable for standard crops. Arguably, such feedstocks have advantages over crop residues, since removal of stover, bagasse, straws, or other residues from fields could lead to soil erosion and nutrient depletion reducing soil productivity [31,32]. Some non-agricultural residue cellulosic feedstock options are abundant and fast-growing (e.g., bamboo). Yet, these are largely ignored due to characteristics of their lignocellulosic matrix.

Lignocellulose

Lignocellulose is composed of three principal components: cellulose, hemicellulose, and lignin (Figure 1). Cellulose is an unbranched, linear polysaccharide generated by the polymerization of more than 10,000 cellobiose subunits, which are simply two β–linked D-glucose molecules [33]. Each cellulose chain is polar with two "end groups": the reducing end features a closed ring structure while the non-reducing end terminates in a carbonyl group [34]. The majority of cellulose is crystalline, a feature that naturally protects the plant from fungal and bacterial attack and that also resists enzymatic breakdown during pretreatment [35]. In crystalline cellulose, cellulosic chains are arranged into parallel microfibrils compacted into dense hydrogen-bonded fibers. This high-density, ordered biomolecular matrix results in a three-dimensional structure that is virtually impermeable to enzymes and, in most cases, even water [36]. From the perspective of plant-pathogen interactions, this is protective against many would-be bacterial, fungal, and viral agents while allowing the plant to strictly regulate water and nutrient exchange. However, from a cellulosic bioethanol feedstock perspective, it presents a highly recalcitrant material. The more recalcitrant the material, the more harsh the chemical pretreatment must be or the more costly the enzyme-mediated deconstruction process will be. Naturally amorphous cellulose (or crystalline cellulose that is made amorphous via pretreatment), lacks the same parallel fiber structure and the dense hydrogen bonding found in crystalline cellulose. This results in a less dense matrix with more accessible

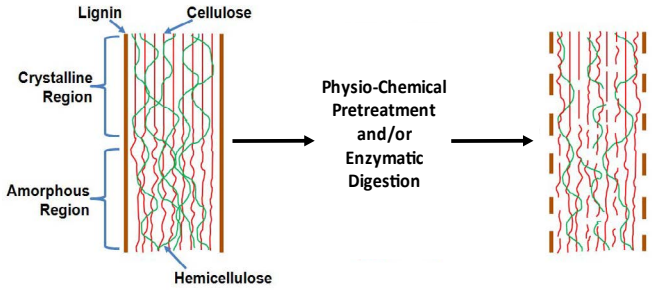


Figure 1: Schematic of basic lignocellulose macromolecular structure before and after pretreatment. Natural lignin (a.k.a., protolignin) *(left)*. Pretreatment *(middle)*. Resulting breakdown of the lignocelulosic matrix after pretreatment (right) adapted from [15].

surface area (and end groups), thereby making it more susceptible to enzymatic degradation. While some enzymes specialize in attacking internal cellulose structure, others attack either the reducing or non-reducing ends of cellulose fibers. Still, lignocellulose deconstruction is not simply a matter of reducing crystalline cellulose to a more amorphous state. Crystallinity is not the only factor that contributes to the recalcitrant nature of lignocellulosic biomass. Bonding between hemicellulose and cellulose and the bonds between lignins and these polysaccharides also significantly contribute. Indeed, bonding with lignin is considered equally problematic as cellulose crystallinity. Lignin disruption is the primary objective when employing harsh acid or alkaline washes during pretreatment. The amount of lignin, the type of lignin, and the ability to remove lignin from the matrix has been a key factor in feedstock and pretreatment selection (*discussed below*). As shown in Table 1, composition of cellulose, hemicellulose, and lignin can vary greatly. It is important to note that percent composition lignin is not a suitable quick measure to select (or exclude) a particular feedstock. Indeed, two different feedstocks with similar percent lignin per unit biomass may exhibit very different levels of recalcitrance due the type of lignin and the bonding pattern of lignin within the matrix.

In contrast to cellulose, the structure of hemicellulose is non-crystalline and highly-branched. Hemicellulose is a polymer of 5-carbon and 6-carbon sugars classified into five main groups: galactomannans, glucomannans, xylans, xyloglucans and β-D-glucans [37]. There are many subtypes of hemicellulose. The chemical and molecular composition of hemicellulose varies considerably depending upon the source. Whereas, some hemicellulose has a glucose backbone, others feature a mannose backbone. In either case, branches off of the backbone feature side-chain complements that are characteristic of the feedstock.

Hemicellulose generally plays a role in forming inter-fiber bonds and strengthening the cell wall. In comparison to cellulose, hemicellulose is a relatively weak, random, amorphous heteropolymer and is easily separated through hydrolysis and enzyme-mediated processes [38]. However, depending upon the type of hemicellulose and how it is structured within the lignocellulosic matrix, it may significantly contribute to recalcitrance.

Lignin is the second most abundant polymer in the world. Lignin is the third component in lignocellulose. Lignin, however, is not a polysaccharide and thus cannot be converted into fermentable carbohydrates. Lignin is an aromatic heteropolymer composed of three main monomers: *p*-hydroxyphenol (H), guaiacyl (G), and syringyl (S)-with varying degrees of methylation (Figure 2). It is the nature of lignin and its bonds to hemicellulose and cellulose that determine how difficult it will be to breakdown lignocellulose. Increasing the number of bonds within lignin (i.e., between lignin subunits) and between lignin and holocellulose per unit biomass results in a more recalcitrant lignocellulosic material. Increased methylation - specifically, an increase in methoxy groups around a phenol results in a decrease in the number of bonds with phenol groups [2,39]. Lignin is typically more difficult to extract than cellulose because of its varied chemical composition - in other words, its high degree of heterogenity [40].

Natural lignin (Protolignin)

As a hydrophobic polymer, lignin offers structural support to cell walls [41]. Lignin forms an evaporation barrier and it has a critical role in channeling water throughout the plant. It protects against parasitic and enzymatic attack by reducing porosity [41]. Lignin emerges from enzymatic dehydrogenative polymerization of three phenylpropanoid precursors: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Figure 2). As the only complex aromatic heteropolymer in the cell wall, lignin contributes to the matrix by forming lignin-carbohydrate complexes. Lignin, via its various subunits, engages in multiple types of bonding arrangements. Both ether (non-condensed) linkages and carbon-carbon (condensed) linkages bind lignin precursors forming networks of lignin-lignin and lignin-polysaccharide bonds within the native lignocellulose matrix. Unfortunately, the structure of native lignin is difficult to elucidate because methods for preparing lignin for analysis disrupt or modify original bonding patterns in the lignocellulosic matrix [42]. Indeed, natural lignin (a.k.a., protolignin) is invariably altered during lignin extraction processes. Neither mechanical nor chemical separation of lignin is possible without modifying the protolignin or introducing hemicellulose or carbon contaminants into the sample. Without readily available ways to determine the impact of particular lignin networks on the deconstruction efficiency of lignocellulosic substrates, the general rule has been to simply avoid feedstock with high lignin content or complex lignin structures.

More recently, however, the aversion to lignin has been tempered. First, it was determined that lignin removed from biomass can be used as a combustible in power generation for ethanol production facilities [43-46]. Second, not all lignin removal results in combustible lignin; in some cases, select extraction methods will modify lignin into valuable lignin byproducts, which may serve as salable co-products to reduce or offset production costs [43,45,47].

Chemical-based lignin extraction and lignin-derived products

Although altered during extraction, natural lignin from fresh feedstock can yield chemically-altered "industrial" lignins [48]. These lignins are the by-products of phenolation, methylation, demethylation, hydrolysis and other modifications to protolignin [41]. Once industrial lignins are extracted they can be further modified to form secondary products such as additives for concrete products, dyes, epoxies and thermoplastics. It is not only the type of protolignin (and thus feedstock) that is important in determining what type of modified lignin product might be derived but also the manner in which the lignin is extracted. Oxidative delignification chemistry and catalysts for lignin

Source	% Cellulose	% Hemicellulose	% Lignin	Refs
Corn Cob[a,b]	32-46	35-40	7-15	[14,180]
Corn Stover[a,c]	35-40	21-26	11-19	[172,180]
Sugarcane Bagasse[a,c]	25-45	25-32	10-25	[172,180]
Barley Straw[c]	36-43	24-33	6-10	[172]
Rice Straw[d]	32-47	19-27	5-24	[181]
Rice Husk[a]	29-36	12-29	15-20	[180]
Oat Straw[a]	31-35	20-26	10-15	[180]
Wheat Straw[a,b,c]	33-39	22-30	9-16	[14,172,180]
Wheat Bran[a]	11-15	36-39	8-13	[180]
Hardwood Stems[a,b]	40-55	24-40	18-25	[14,180]
Softwood Stems[a,b]	45-50	25-35	25-35	[14,180]
Barley Husks[a]	34	36	19	[180]
Cotton Stalks[a]	59	14	22	[180]
Sunflower Stalks[a]	42	30	13	[180]
Pine[a]	45	22	28	[180]
Miscanthus[c]	43	24	19	[172]
Switch grass[a,b,c]	32-45	25-31	12-20	[14,172,180]
Bamboo[c]	49-50	18-20	18-28	[172]
Moso Bamboo[c]	37-47	22-23	23-31	[172]

Table 1: Molecular composition of select lignocellulosic substrates.

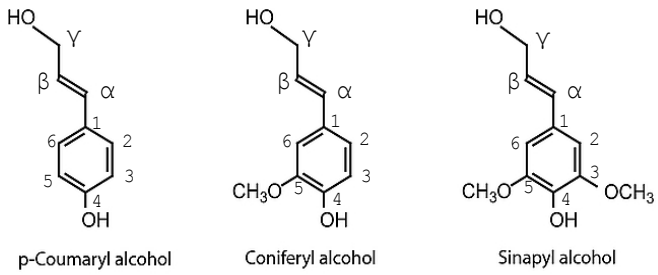


Figure 2: Lignol Structures. Chemical structure of lignin alcohol precursors, the lignols, that incorporate into lignin as phenylpropanoids. The three main lignin phenylpropanoids are: *p*-hydroxypheyl (H), guaiacyl (G), and syringyl (S).

oxidative conversion have been recently reviewed, including those techniques used in the pulp and paper industries [49]. Below are brief descriptions of select lignin extraction processes. Many are common to the pulp and papermaking industries and the bioethanol industry.

Dilute-Acid Lignin (DAL): Using dilute acid to separate lignin from biomass is the most basic and most common extraction method. An acid wash (or a sequence of multiple acid washes) at elevated temperatures (e.g., 130˚C-210˚C) will breakdown xylans and disrupt interactions between hemicellulose and lignin [36,50]. The most commonly used acid for such pretreatments is dilute sulfuric acid (H_2SO_4), which is used in commercial operations for the pretreatment of several different feedstock types [51]. Other acids are also employed, including: hydrochloric acid (HCl) [52], nitric acid (HNO_3) [53] and phosphoric acid (H_3PO_4) [54]. The resulting lignin is a low molecular weight solid.

Steam-Exploded Lignin (SEL): Steam explosion followed by enzyme-mediated hydrolysis is another of the more common treatments for lignocellulosic biomass. High yields of both lignin and desired polysaccharide substrates are recovered from this extraction method. The resulting lignin fraction is generally a solid [55]. Although multiple feedstock types are processed via steam explosion (resulting in different lignin byproducts), most steam-exploded lignin (especially from woods) exhibit increased carbon-carbon inter-unit linkages, slightly lower methoxy group composition and increased phenolic hydroxyl group composition than the protolignin from which it is derived [49,55,56].

High-Pressure Refining Lignin (HPL): High-pressure refining lignin is produced by treating wood biomass (i.e., wood chips) with dilute sulfuric acid and a series of pressurized (145-218 psi) high temperature (178-198˚C) steam treatments followed by enzymatic hydrolysis [57,58]. The resulting fibrous pulp emerges as a solid and the lignin structure resembles SEL [49].

Soda Alkaline Lignin (SAL): "Soda pulping" employs an alkali solution at elevated temperatures (170˚C) to begin the biomass deconstruction process [59]. Soda pulping results in a slurry of black liquor and pulp from which cellulose is extracted and lignin is separated out. This separation is often conducted using a H_2SO_4 wash followed by a water rinse and subsequent oven-drying to precipitate out and produce the final lignin product [58].

Pyrolytic Lignin (PYL): Using pyrolysis on lignocellulosic biomass results in the generation of a pyrolytic oil of which lignin constitutes part of the liquid fraction. Using phase separation techniques, the lignin can be removed from the rest of the bio-oil [60]. The resulting product is a relatively low-molecular weight eight-carbon (C_8) backbone structure known as pyrolytic lignin [61]. Side chains extending from the basic structure vary but form carbon-carbon inter-unit linkages are prevalent. The exact nature of such linkages depends upon the specific thermal treatment that is applied. Pyrolytic lignin is considered less stable than other lignin products and thus amenable to downstream modification to yield other valuable lignin products [49,62].

Organosolv Lignin (OSL): Organosolv lignin is extracted using an organic solvent that solubilizes the lignin. After separation from the slurry, the solvent can be evaporated off (or precipitated out) to leave a relatively pure lignin product. Organosolv extractions generally target β-aryl ether bonds and thus preserve much of the primary lignin structure [40]. The resulting lignin is typically characterized by a low-molecular weight and it is soluble in organic solvents [63].

Ammonia Fiber Expansion Lignin (AXL): Addition of ammonia to a high-temperature (90-100˚C) and high-pressure biomass deconstruction process that includes a timed, sudden pressure release will disrupt lignin-polysaccharide bonds in lignocellulose [64]. This will swell the matrix and reduce cellulose crystallinity thus increasing surface area for subsequent enzyme-mediated hydrolysis. The resulting lignin fraction is typically insoluble and the nature of the lignin structure has not been thoroughly described [65]. Since ammonia fiber expansion (AFEX) is a very common lignocellulose biomass pretreatment process in cellulosic bioethanol production, it is reasonable to explore the nature of the resulting lignin byproducts and their potential commercial value.

SPORL Lignin (SPL): Sulfite pretreatment to overcome recalcitrance of lignocellulose (a.k.a., SPORL) yields sulfonated lignins [51,66]. These lignosulfonates are present in both the liquor and the solid fraction of the resulting pretreatment products. These are higher than average molecular weight hydrophilic lignin products [51,67] that can be used for a variety of commercial purposes.

The basic properties and structure of lignin products resulting from the above eight processes are provided in Table 2. These have been highlighted since they are common in bioethanol production processes. There are multiple other extraction processes that have been used in pulp and papermaking industries and/or bioethanol production. Many of these employ similar extraction methods to those described above or combine one or more of these methods. Some of these additional methods result in unique or historically identified lignin products.

For example, Braun's Lignin, Bjorkman Lignin, Klason Lignin, Kraft Lignin, Willstatter Lignin, Cupraxm Lignin, and several other lignin products have been developed from different industrial processes. Although it is beyond the scope of this review to detail each of these, it is important to note those, which are considered to minimally disrupt the lignin from its natural state. For example, Braun's Lignin uses an organic solvent such as benzene-ethanol on woody biomass [62]. Although this method generally has a low yield, the extracted lignin is similar to its original structure within the matrix. Braun's Lignin has a low molecular weight, high phenolic hydroxyl content, high syringyl content, and a high number of ester groups [68]. Another extraction method that leaves native lignin intact is the Bjorkman lignin extraction method, which was developed in 1956 and uses a combination of mechanical pretreatment (i.e., vibratory or rotary ball milling), organic solvent extraction, and enzymes to produce a close to natural lignin product [68]. Although a nearly intact lignin product emerges, the lignin often contains 10% or greater residual carbohydrates as "contaminants" [62]. Klason Lignin (a.k.a., sulfuric acid lignin) is extracted with alcohol-benzene and hydrolyzed with 60-75% sulfuric acid (H_2SO_4). The alcohol-benzene extraction removes

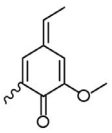
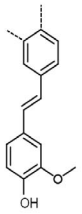
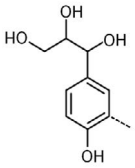
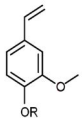
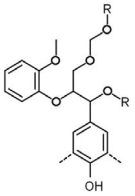
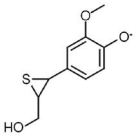
Lignin Type	Extraction Method	Lignin Properties	Chemical Structure of Lignin	Reference
DAL	□ biomass is treated with mineral acid at high temperatures (e.g., 160°C) lignin fraction separated as a solid	□ low carbohydrate content lignin □ partially cleaved ether and ester linkages □ low molecular weight lignin with increased hydroxyl content		[49]
SEL	□ pressurized steam is used to disrupt matrix □ enzymatic hydrolyses remove carbohydrates leaving a solid lignin residual. □ high lignin yield	□ SEL from soft woods exhibit lower methoxy group content and higher phenolic hydroxyl content □ SEL from soft woods exhibit more frequent C-C inter-unit linkages.		[57]
HPL	□ heat and mechanical force produces a fibrous pulp □ lignin is separated via enzymatic hydrolysis	□ HPL emerges as a solid □ HPL resembles SEL	*(same as SEL above)*	[57]
SAL	□ akali wash at high-temp results in a slurry of pulp and black liquor □ lignin is precipitated by lowering pH (pH 2)	□ ionization of one free phenolic OH group at the C4 site synthesizes quinone intermediates and eliminates bonds at the α position		[190]
PYL	□ biomass undergoes pyrolysis and the lignin is converted into a liquid form.	□ PYL has a C8 basic unit skeleton and low molecular weight □ new side chain formations and cracking reactions are observed □ PYL lignin is unstable facilitating new co-product synthesis		[60]
OSL	□ Lignin is dissolved and isolated from the biomass using a high percent organic solvent	□ relatively pure lignin is exhibiting low molecular mass with high phenolic and aliphatic hydroxyl side chains □ Hibbert's ketones may be produced		[63]
AXL	□ Ammonia is added to the biomass under high temperature and high pressure, and the pressure is suddenly released □ High lignin yield after substrate pretreatment	AFEX results in reduced cellulose crystallinity □ lignin-carbohydrate bonds are broken □ high lignin yield but the structure of lignin is not well-defined	*(structures not well-defined)*	[64]
SPL	□ Similar to sulfite pulping, the lignin is dissolved in spent pretreatment liquor and isolated as a solid	□ Higher than average molecular weight and hydrophilicity □ High sulfur content		[44]

Table 2: Lignin Extraction Lignin properties and structure by extraction method.

contaminant resins, waxes, lipids, and wood gums and the H_2SO_4 mediates hydrolysis, thus separating lignin from polysaccharides. Klason lignin closely resembles SAL. Kraft Lignin is produced through a process known as "pulping". Specifically, a strong alkali solution and a sodium sulfide catalyst are used to separate lignin from hemicellulose. This generates what is known as "black liquor". Black liquor can be used as a fuel supplement or to regenerate inorganics used by the mill [69-79].

Black liquor can be further processed to produce other valuable products such as water-reducing agents [40]. The cellulose fraction is processed via "serial bleaching" to make paper pulp. Lignin is also used as a component in dyes, dispersants, and pesticides [80]. Willstatter lignin is extracted from wood meal via hydrolysis with hydrochloric acid (HCl). Although effective for extraction, the final product is an insoluble lignin [81]. Cupraxm Lignin employs cuprammonium hydroxide ($CuH_{14}N_4O_2$) to wood meal to remove carbohydrates from

biomass. This flushing out of carbohydrates leaves a residual relatively pure lignin product [82]. Periodate or "Purves" lignin is also reported to be similar to protolignin. Purves lignin is also extracted from wood meal using a sodium hydroxide (NaOH) soaked at room temperature with subsequent neutralization using hydrochloric acid (HCl) followed by a sodium periodate ($NaIO_4$) treatment [83]. Although there is a high yield of oxidized native lignin, the process is costly and slow. The key reasons for recalling these different processes and lignin products are four-fold. First, different lignin structures emerge from different extraction processes. Second, most lignin production is a byproduct of harsh chemical treatments to lignocellulosic biomass that leave hazardous waste. Third, the lignin product may not only be specific to the extraction method but also to the feedstock used. Fourth, depending on the chemical structure and properties of the emerging product, these lignin products may have significant commercial value.

Economics of lignin

It is unlikely that for-profit companies will harvest feedstock and pretreat lignocellulosic biomass with lignin as the principal product. Indeed, lignin is, has been, and will likely always be a co-product of other industrial operations including bioethanol, paper and pulp production. The adage that "You can make anything out of lignin except money" [49] is still pervasive throughout industry. However, as more and more lignin-based products make it into the market, this sentiment may fade. Tight margins and fluctuations in the level of federal subsidies (at least in the U.S.), drive bioethanol producers to constantly consider ways to reduce or offset costs by producing valuable co-products. Although "yellow cake" and corn oil are dominant co-products for corn ethanol, new uses for lignin in the global market are growing and may help cellulosic ethanol to be more competitive. Table 3 illustrates the diversity of salable lignin products, their uses, availability, and potential market value. When compared to the growing cellulosic bioethanol sector alone (Table 4), it is clear that strategies on the extraction and preparation of lignin for internal use or the market should be a top priority.

Beyond the lignin-based products describe in Table 3, lignin is also used as a binding agent in makeup, lotion, and other aesthetic care products [84]. Recent research also suggests that certain lignin-based compounds may have medicinal value. This includes potential anti-cancer properties [84], anti-viral properties [18,85], and perhaps a role in regenerative medicine [86]. Although substantial research needs to be completed to validate initial reports on anti-cancer and anti-microbial bioactivity, the possibilities for lignin engineering and novel applications appear to be broad. For this reason, feedstock with high lignin-content or with complex lignocellulosic structures should not be automatically discounted, especially if they have other desirable properties such as rapid growth and high cellulose density.

Environment-friendly lignin production

With diminishing natural resources and continuous fluctuations in the availability of low cost transportation fuels, alternative sources of fuel are being explored. These, of course, include the "biofuels" such as bioethanol. Arguably, it is the bioethanol industry that may have greatest access to lignocellulosic biomass. As an environment-conscientious industry, bioethanol producers have the reputation of leaning towards environment-friendly biomass processing as long as it does not interfere with profit margins. The need to pretreat feedstock with hazardous materials is one of the key obstacles to expanding the cellulosic ethanol market to the same level as corn- and sugarcane-based ethanol. Indeed, producers and research groups alike are ardently exploring ways to deconstruct lignocellulose while minimizing the amount of treated and hazardous waste produced during the process. Interestingly, there is a nexus in the "clean" and efficient use of polysaccharide for bioethanol production and the extraction of salable lignin whereby abiotic and/or biotic biomass treatments may play an important role in producing environment-friendly products (and co-products). It is at this intersection in technologies that the development of novel strategies may open the industry to feedstocks that have been previously ignored or understudied for large-scale production.

Alternative cellulosic feedstocks

The most common feedstocks for cellulosic ethanol are those associated with agriculture or pulp and papermaking. These include: corn stover, sugarcane bagasse, wheat straw, rice straw, and wood biomass. A few producers use municipal waste and non-agricultural grasses or other C_4 plants. These latter non-agricultural cellulosic biomass sources may be considered "alternative" SGB feedstocks. Indeed, several studies have investigated the potential use of *Sorghum* as an alternative SGB feedstock [69-72]. Likewise, switchgrass (*Panicum virgatum*) has been studied as a viable non-agricultural SGB feedstock [73-75]. Recalling that one of the key considerations in determining whether a feedstock is viable for bioethanol production is the nature of its lignin structure, it should be noted that such grasses typically polymerize lignin from all three types of monolignols in roughly equal proportions and that lignin subunits are often linked by non-hydrolyzable ether and alkyl bonds [2,39]. Ferulic acid bridges ether-bond to lignin and ester-bond to the arabinofuranosyl residues of hemicellulose resulting in highly recalcitrant matrix structures [76]. Other bonding events such as ester-bonded uronyl bridges and other linkages between lignin and hemicellulose (e.g., to hemicellulosic glucuronoxylans) further complicate and strengthen the matrix [76,77]. However, it is not only the linkages between lignin and hemicellulose that inhibit access to cellulose (and the separation of lignin). Depending on the feedstock, there may also be significant bonding between lignin, hemicellulose, and cellulose beyond simple cellulose-to-hemicellulose hydrogen bonding. Although direct bonds between cellulose and lignin tend to be more elusive, it is known that lignin can form ester linkages with hemicellulose, which in turn can covalently bond to cellulose [78]. Bonding between all three components further strengthens the matrix making it even more recalcitrant. It is the complexity of the lignocellulosic matrix of each feedstock as well as feedstock abundance/availability that ultimately determines if a feedstock is economically viable for input into a bioethanol production.

The potential for bamboo as a SGB feedstock

Arguably, one of the most underestimated alternative SGB feedstocks is bamboo. Like Sorghum and switchgrass, bamboo is a grass. It is a large, perennial forest grass naturally distributed worldwide except in Europe and parts of North America. Bamboo is a vernacular term for members of the subfamily *Bambosoideae* [87,88] of the grass family *Poaceae* otherwise known as *Germineae* [89]. There are three tribes of the subfamily Bambusoideae: Bambuseae, Arundinareae, and Olyreae [90]. Olyreae is the least renowned of the three tribes. Characterized by shorter and weaker culms, this herbaceous tribe is less important economically than the woody species and therefore has received less attention from the scientific and business sectors. Bambuseae and Arundinareae are the tropical and temperate woody tribes, respectively. They are the archetypal species of "bamboo" featuring tall, green, and robust lignified culms that can be used as construction materials due to their woody properties [91]. They play critical roles not only in their respective ecosystems but also in local economies.

Product	Lignin Substitute	Industry	Uses	Distribution	Value
Phenolic resins	acetosolv lignin OSL DAL	phenolic resin	circuit boards adhesives	8.25 M tonnes (2004)[§, [80]]	$107 billion (2005)[§, [80]]
Concrete admixtures	Lignosulphates	concrete production	binding agents dispersants	1.0 M tonnes (2001)[§, [80]]	$18.26 billion (by 2019)[§, [183]]
Kraft lignin	Kraft Lignin soda Lignin	agriculture fashion	pesticides dispersantsdyes	100,000 tonnes (2004)[83]	$300 million (2004)[83]
Epoxies	Kraft lignin Alcell lignin SAL SEL other lignins, which react with epicholohydrin	electrical engineering	printed circuit boards (IBM)	350kL/ann.[§, [80]]	$2 Billion* (1999)[§, [80]]
Ahesives	In situ Lignin cross reacted with laccases	fiberboard	housing construction	16.8 Mm^3 tonnes (2006)[§, [80]]	n/a
Polyolefins	Epoxy modified lignosulfunate in abinary polyoefin mixture, Kraft, or other animated lignins	thermoplastics	clothing sports equipment	63.4 M tonnes (2006)[§, [80]]	$19 Billion (2001)[§, [80]]
Polypropelene	Kraft and other animated lignins	thermoplastics	packaging labeling containers	38 M tonnes (2005)[§, [80]]	$42 Billion (2005)[§, [80]]

Table 3: Select industrial products for which there is a lignin-derived substitute or additive. [§]global distribution and dollar value; *Japan, US, and Western Europe cumulatively; n/a – Not available.

Company	Feedstock (s)	Technology	Output	Comments	Source
Abengoa Hugoton, KS, USA	corn stover, wheat straw, prairie grass	enzymatic digestion	25 million GPY	burns lignin for power co-produces electricity opened late 2014	[a]
Abengoa Salamanca, Spain	wheat straw barley straw	enzymatic hydrolysis	1.3 million GPY	started production 2008	[30]
Alpena Biorefinery Alpena, MI, USA	hardwoods	Hot water extraction, acid hydrolysis	945,000 GPY	demonstration plant opened late 2013 co-produces energy and 700K gal of CH_3CO_2K	[206]
Beta Renewables Crescentino, Italy	bagasse wheat straw rice straw giant reed	steam explosion, enzymatic digestion	20 million GPY	burns lignin for power co-produces electricity recycles 100% of water	[30]
BlueFire Renewables Fulton, MS, USA	wood pellets	sulfuric acid hydrolysis	19 million GPY	burns lignin for power	[b]
BlueFire Renewables Lancaster, CA, USA	municipal wastes	sulfuric acid hydrolysis	3.9 million GPY	burns lignin for power	[b]
Borregaard Sarpsborg, Norway	spruce wood	sulfite pretreat enzymatic hydrolysis	19 million GPY	produce >30 co-products (e.g., vanillin and lignin)	[30], [207]
Chempolis/NRL Assam, India	bamboo	NA*	NA*	expect completion 2017	[c]
DuPont Biofuel Solutions NV, IA, USA	corn stover	ammonia steam explosion, enzymatic digestion	30 million GPY	burns lignin for power, co-produces electricity	[d]
GranBio Alagoas, Brazil	sugarcane bagasse, straw	steam explosion, enzymatic digestion	22 million GPY	opened September 2014 burns lignin for power co-produces electricity and vinasse fertilizers	[e]
POET-DSM Emmetsburg, IA, USA	corn stover corn cobs	enzymatic	20 million GPY	will expand to produce 25 million GPY	[f]
Raizen Energia Piracicaba, São Paulo, Brazil	bagasse straw	dilute acid steam pretreatment; enzymatic hydrolysis	22 million GPY	next to sugarcane mill co-produces electricity	[g],[h]
Zeachem, Boardman, OR, USA	stover, cobs, hybrid poplar	physio-chemical; enzymatic hydrolysis	25 million GPY	demonstration plant opened in 2014	[30]

[a]www.abengoabioenergy.com/web/en/2g_hugoton_project/; [b]www.bfreinc.com/our-technology/;
[c]www.nrl.co.in/newscentre.asp?pg=main&lnk=press; [d]www.biofuels.dupont.com/cellulosic-ethanol/;
[e]www.granbio.com.br/en/; [f]www.poest-dsm.com/pr/first-commercial-scale-cellulosic-plant;
[g]www.iogen.ca/raizen-project/; [h] http://www.iogen.ca/cellulosic_ethanol/index.html;
***NA**=data not available; **GPY**=gallons per year.

Table 4: Selected cellulosic ethanol production plants from around the globe.

Species of Bambuseae and Arundinareae are characterized by "gregarious monocarpy" [92], which is a single flowering season that can occur from a few years to 120 years after shoot emergence. Bamboo is known as a fast growing plant that readily propagates and that has high mechanical strength properties. This renders bamboo as an ideal raw material for the manufacture of furniture, ply bamboo, and the construction of common structures such as rural houses and walking bridges [93]. There are over 1000 species of bamboo within ~100

genera distributed globally with the largest concentrations in tropical and sub-tropical regions [94-96]. Out of these, only 58 species are used to produce wood-based products, while 18 species are used to extract fiber for composite materials [97]. In spite of use by rural communities in many countries for housing, food, bridges, musical instruments, and more as well as larger-scale commercial endeavors in furniture and composite flooring manufacturing, bamboo has not been utilized as a principal feedstock for bioethanol production. Although there is not a single major cellulosic ethanol plant in the world that uses bamboo as a primary feedstock, one is under construction in India, which is anticipated to be operational in 2017 (Table 4). With the exception of one of the largest bamboo species (Moso bamboo), most bamboo species have lignin content (18-28%) on par with many other well-utilized SGB feedstocks such as corn stover (11-19%), bagasse (15-25%), wheat straw (12-16%), and rice straw (17-19%) (Table 1). Furthermore, the cellulose content in bamboo can be as much as 50% by composition, which exceeds that of other currently used SGB feedstocks. A review of the lignocellulose characteristics of the family Poaceae indicate that the cell wall properties of bamboo culm are not significantly different from other grasses, including those used as principal SGB feedstocks. Long cellulose microfibril chains [98], the enrichment of aromatics [99], and/or the presence of ester and ether cross-linked glucuronoarabinoxylans between lignin and matrix polysaccharides [100], may render bamboo lignocellulose recalcitrant. However, bamboo lignocellulose (on average) is not any more recalcitrant than other mainstream SGB feedstocks (e.g., bagasse). Thus, the type of lignin in bamboo should also not be a concern for considering bamboo as a bioethanol feedstock. Rapid growth, the ability to grow in low-nutrient soils, abundance, broad distribution, low-ash content, high cellulose composition, high biomass density/low moisture content, and the fact that it does not need to be replanted upon harvest as new shoots sprout from the rhizome [101,102] are all properties that make bamboo an ideal candidate as a SGB feedstock.

Historically bamboo has been largely ignored as a prime feedstock for bioethanol production, especially by the western world. It has been suggested that drawbacks in selective breeding of a bamboo, a lack of understanding of bamboo reproductive physiology (i.e., flowering), and limited experience with bamboo propagation in the west may underlie the lack of attention that bamboo has received as a potential SGB feedstock [103]. However, more recent studies suggest that the use of bamboo as a SGB feedstock is both technically and economically feasible, especially in certain regions of the world, including Southeast Asia [104]. Furthermore, rural community interests in developing bamboo plantations and recent work with propagation strategies may result in a resurgence of enthusiasm for bamboo-based bioethanol. One promising strategy is to use spent oil palm fields, which are nutrient-depleted, to propagate hardy native bamboo species in Malaysia (particularly in Sarawak on the island of Borneo). More than 3.6 million hectares (ha) of oil palm is planted in Malaysia (and 4.1 Mha in Indonesia) [105] and yet this energy crop leaves soil so nutrient-depleted after the cultivation cycle ends that further use for oil palm is not feasible and other food crops will not readily grow.

Instead of leaving large tracts of land barren, development of bamboo plantations may be an option. Whether bamboo can compete with western SGB feedstock staples such as stover, bagasse, and straws remains to be seen. The chemical profile of bamboo and above-ground net primary productivity is on par with or exceeds other primary SGB feedstocks. For instance, under optimal conditions, some large bamboo (e.g., *Gigantochloa Levis*) can grow to 20 m with 10 cm diameter culms and 2.5 cm thick highly dense/low moisture content culm walls [101,102]. Recent studies cite annual holocellulose production of bamboo as >8 times that of corn stover [106]. Perhaps the only impediment to establishing bamboo as a principal SGB feedstock is lack of motivation. The USDA concluded that other crops could out-produce bamboo under the majority of environmental conditions in the U.S. and that land not already in use for food production in the U.S. was unsuitable for cultivating bamboo [103]; however, these assertions do not appear to be supported by scientific data and no large-scale experiments were ever performed to confirm such claims. Although most species of bamboo require a mean annual temperature of 15-20°C, they are hardy and some species can survive lows of -20°C without any detriment to growth and survivability [89]. Indeed, many species of bamboo are fast-growing, cellulose-rich, low-moisture content, productive plants that can grow in a variety of soils and under a variety of environmental conditions. The only absolute environmental need would be precipitation. Bamboo is not a C_4 plant and typically requires 100-150 cm of mean annual precipitation. This indeed could limit cultivation of bamboo in regions with little rainfall. Still, reconsidering the potential role of bamboo as a major SGB feedstock may be justified.

In addition to high cellulose content, up to 20% of total sugar mass may be xylose [104]. Specifically, the hemicellulose component of bamboo is dominated by glucuronoarabinoxylan (as noted by the frequency of arabinose side-chains, as well as methyl groups, acetyl groups, sugar-acid moieties branching off the xylan backbone), which is characteristic of most grasses, including bagasse [37,107]. Xylose can be directly fermented to ethanol. High xylose content, in this case, can be an advantage - especially, if the species is still harboring up to 50% cellulose in its lignocellulose. Despite these advantages, bamboo is also commonly dismissed due to its high lignin content; however, it is clear that lignin content (even in fully-lignified bamboo) is on par with other principal SGB feedstocks and the fact that a culm can reach full height before becoming completely lignified is commonly ignored.

In bamboo, the composition of holocellulose does not change from the time it develops a shoot to the time it fully matures and lignin does not begin to accumulate in the bamboo to any appreciable degree until *after* the culm reaches its full height [76]. After reaching full height, lignification then proceeds independently within each internode from top to bottom and from inside to outside. Full lignification is complete within one growing season and the lignin remains constant throughout the life of the culm. The lignin in mature bamboo is not vastly different from that of bagasse or wheat straw since all three of these lignocellulosic feedstocks are grasses. Lastly, the cultivation and use of bamboo as a SGB feedstock would not contribute to the *food vs. fuel* dilemma. (Notably, most species used as food are not the same as those that would be used as SGB feedstock).

In spite of all of the aforementioned advantages, like other SGB feedstock, bamboo requires processing to deconstruct its lignocellulosic matrix to expose polysaccharides to sugar-reducing enzymes. Such pretreatment will require the separation and/or removal of lignin.

Bamboo lignin

The bamboo cell wall consists primarily of hemicellullose, cellulose, and lignin. It has been reported that holocellulose and lignin make up 90-98% of the composition of the cell wall with the remaining 2-10% consisting of a variety of extractives, resins, tannins, waxes and inorganic salts [174,177,178]. These components ultimately make up the physical structure of bamboo – the epidermis, parenchyma cells, vascular bundles, and supporting fibers that tie these together in a hierarchical structure [179]. Cellulose microfibrils traverse and intertwine densely through the lignin-holocellulose matrix (Figure 3). These linear chains of glucose form an extensive network of

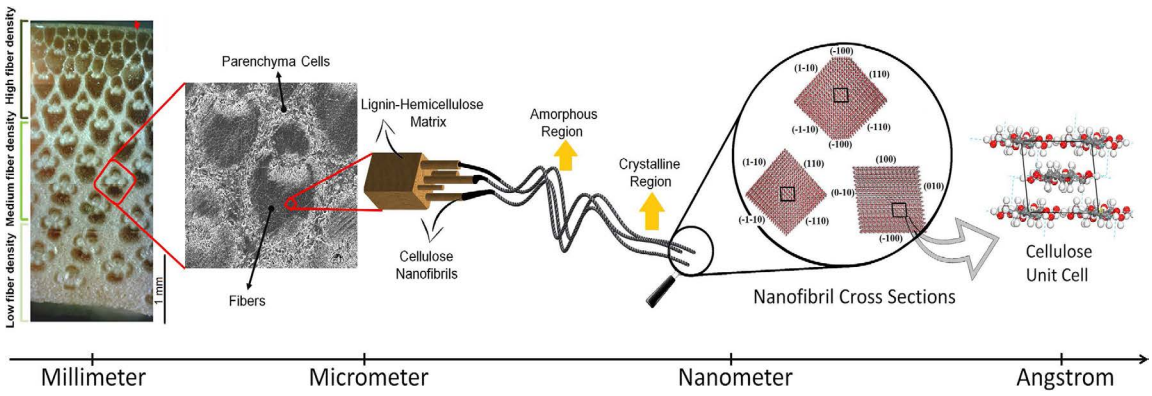


Figure 3: Hierarchical structure of bamboo [179].

hydrogen bonds throughout the matrix. Those regions that consist of highly ordered hydrogen bonding are *crystalline*; whereas, those less ordered regions are more *amorphous* by definition. The level of cellulose crystallinity in lignocellulose impacts the efficiency of enzyme-mediated degradation and overall biomass recalcitrance. For highly-ordered crystalline cellulose, cross-sections through the matrix (i.e., perpendicular to parallel cellulose fibers) show rectangular or hexagonal profiles (Figure 3).

It has been suggested that the macromolecular order of the fibrous structure within bamboo contributes to the remarkable mechanical properties of the culm [180,181]. Indeed, it appears that the geometry of the cellulose fiber networks along with the fact that they are embedded in a matrix of lignin and hemicellulose endows bamboo with favorable properties, such as a high strength-to-weight index [182]. The bamboo culm composite is approximately 74% cellulose by volume with ~13% hemicellulose and ~11% lignin [183]. Like percent composition by mass (Table 1), these values may vary depending upon species. (Note that moisture content plays a key role in determining percent composition by volume).

As discussed in previous sections, an important consideration in the use of any potential feedstock for biofuels is biomass lignin content. Feedstocks with reduced lignin content are more readily deconstructed; however, lignin also supports the growth of fiber-dense (and large) plants and thus significant amounts of cellulose. For bamboo, it is important to consider the role that lignin plays in the strength of the culm. Specifically, how necessary is lignin for larger diameter, tall culm growth? And, what are the relative contributions of cellulose, hemicellulose, lignin, and the interactions between these constituents to culm strength, growth rate, culm wall thickness, and maximum culm height? Relative contributions of macromolecular components to material strength may be estimated using molecular models by examining the prevalence of select bonding types within the material.

For example, radial distribution functions (RDF) may be generated to assess the relative contribution of cellulose, hemicellulose, lignin, and bonds between these macromolecules (e.g., hemicellulose-to-lignin) to the overall mechanical strength of bamboo [179]. Using molecular modeling and dynamics methods (i.e., atomistic simulations), Youssefian and Rahbar demonstrated that C-H bonds are most abundant in bamboo lignin and C-C bonds are more abundant than O-H bonds (Figure 4). Furthermore, the number the number of O-H bonds and C-C bonds are similar in hemicellulose. These data suggest that, with respect to strong bonding motifs, C-H bonds in lignin and lignin complexed to carbohydrates dominate the interatomic bonding landscape in bamboo culm.

In Figure 4, distances between non-covalently bonded hydrogen and oxygen atoms are denoted as non-bonded interactions (left peak). Distances between two non-bonded carbon atoms (e.g., C_1 and C_3) in a cyclohexane ring separated by a single carbon atom to which both are bonded (e.g., C_1-C_2-C_3) are also represented by non-bonded interactions (right peak). By comparing the abundance of hydrogen bonds against the abundance of O-H bonds, it is argued that most hydroxyl group hydrogens are engaged in hydrogen bonding in lignin. However, in hemicellulose the contribution of hydroxyl groups to hydrogen bonding is only ~60% [179]. Lignin also exhibits greater hydroxyl group-mediated hydrogen bonding than those in LCC or hemicellulose. Furthermore, hydroxyl groups from hemicellulose are associated with xylose groups and do not extend out from main chains as do hydroxyl groups in lignin. Therefore, it is argued that these and other ultrastructural features endow lignin with a greater capacity to form hydrogen bonds. At a density of approximately 1.3 g/cm^3, bamboo lignin has a Young's modulus, ranging from 2-6 GPa. It is adhesion energy to the cellulose microfibril networks is ~ 150 mJ/m^2. All of these features together highlight the significant role of lignin in the structural integrity of bamboo culm.

Bamboo lignin is composed of all three of the primary phenylpropanoid lignin subunits (i.e., H, G, and S), which are part of a host of polymerization reactions during lignin biosynthesis [183]. As occurs with other feedstocks, lignin extracted from bamboo may vary depending upon the extraction process employed [184]. Indeed, common industrial names such as "milled wood lignin", "dissolved lignin", and "alkaline lignin" are so named because of the extraction processed used, not the feedstock type.

Still, studies using techniques such as Uv-Vis, FT-IR, ^{13}C NMR, HSQC, and other methods, demonstrate that lignin extracted from a single feedstock by different pretreatments and lignin extraction processes may retain core structural properties. For example, it has been demonstrated that the FT-IR spectra of milled wood lignin (MWL), dissolved lignin (DL), and alkaline lignin (AL) from a single species of bamboo are almost identical [185]. Likewise, ^{13}C NMR spectra indicate that MWL, DL, and AL from a single bamboo species were all HGS-type lignin with partial acylation (<12%) at a specific side chain γ-carbon [185]. However, the same study also reported that the level of β-O-4 coupling and the molecular weight of the lignin products varied – specifically, from highest molecular weight to lowest, an AL>MWL>DL pattern resulted. It was also

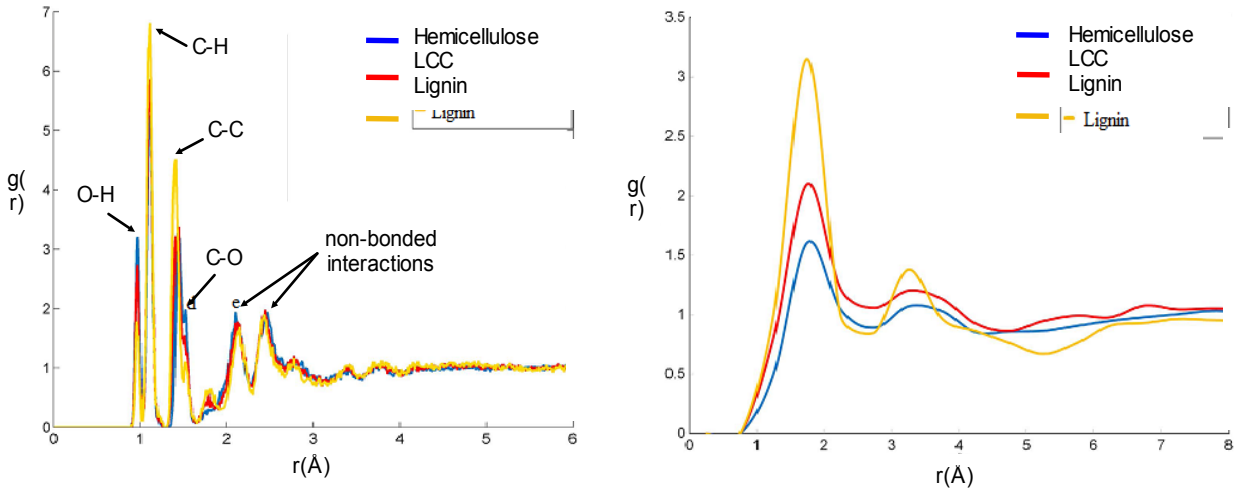


Figure 4: Molecular Modeling of Bamboo Ultrastructure. (A) Radial distribution functions of all atoms in hemicellulose, lignin-carbohydrate complex LCC and lignin showing the first four peaks related to covalent bonds of O−H, C−H, C−C and C−O, respectively. A fifth peak and sixth peak exist due to the non-bonded interactions in the systems. (B) Radial distribution functions between hydrogen atoms of hydroxyl groups and the oxygen atoms in hemicellulose, LCC, and lignin showing the first peak at 1.85 Å related to hydrogen bonds and the second peak at 3.25 Å is attributed to the oxygen-oxygen distance on two hydroxyl groups that are bonded by hydrogen bonds [179].

reported that there were differences in frequency of LCC linkages, acetylated β-O-4, β-β, β-5 and S/G ratios. Thus, depending upon the method of extraction used to isolate lignin at least minor changes in structure may emerge since each process modifies lignin polymers in a different manner. And, of course, independent of structure, lignin yields vary depending upon the extraction process employed. For example, in the same study, MWL processing of feedstock resulted in 18.26% yield, while DL and AL processing yielded 25% and 66%, respectively.

For practical purposes, the question of whether different methods of lignin extraction produce distinct lignin products for a given bamboo species is not as critical as asking, "Which extraction method on which species of bamboo yields valuable lignin co-products?" As described in previous sections, lignin is typically a co-product resulting from other industrial processes (e.g., bioethanol production). These processes often employ pretreatments, which may impact the type of lignin produced in a way that differs from processes where lignin is the primary product of interest. Organosolv pretreatment has been touted as a pretreatment that yields high-purity, low molecular weight and sulfur-free lignin that may be used for the production of value-added products [40,63]. However, using an ethanol organosolv pretreatment on culm biomass from *Dendrocalamus brandisii*, 2D-HSQC NMR data indicated that lignin primary structure can be disrupted via organosolv pretreatment despite the fact that some lignin primary structures and lignin functional groups are preserved through the pretreatment and lignin removal process. Without pretreatment of *D. brandisii*, Klason lignin was produced at a yield of ~24%. However, adding the ethanol organosolv pretreatment reduced that yield to ~17% [40]. In addition, twelve lignin substructures emerged from the 2D-HSQC NMR spectral analysis of pretreated biomass, two of which had sub-forms with additional functional groups (Figure 5). In spite of the HSQC and FT-IR data which suggested that processing options do not significantly change the final lignin product, these 2D-HSQC NMR data indicate that, at least in the case of ethanol organosolv, pretreaments can result in additional polymer breakdown (i.e., cleavage of β-aryl ether bonds), production of additional lignin products, and a reduction in overall yield of the desired lignin co-product (e.g., Klason lignin). Nonetheless, the prevalence of aromatic lignin structures emerging from extraction processes provides an attractive opportunity to convert lignin into valuable aromatic hydrocarbons and other co-products. Cumulatively, these data suggest that more investigation is needed to elucidate the types of lignin products that are produced when biomass from select species of bamboo are pretreated by distinct methods.

Feedstock pretreatments

Regardless of the feedstock, chemical disruption of lignin bonds within almost any lignocellulosic matrix is quite feasible using one of the multiple extraction methods mentioned previously. However, simply breaking the bonds between lignin and polysaccharide is not sufficient. The simple presence of lignin within the slurry can inhibit enzyme-mediated hydrolysis via irreversible binding of lignin to enzymes preventing enzymatic action on matrix polysaccharides [106]. If the end goal is to use a feedstock for bioethanol production, the biomass has to be optimally prepared for enzyme-mediated hydrolysis of cellulose and other convertible polysaccharides. Complete lignin breakdown or removal is vital to most operations. Pretreatments for preparing bioethanol feedstock for subsequent enzyme-mediated processes include many of the lignin extraction methods described above. In short, pretreatment liberates the cellulose (and hemicellulose) components of the biomass so that they may be further broken-down into glucose or other fermentable sugars for yeast-mediated conversion to bioethanol.

Lignocellulosic biomass (i.e., feedstock) pretreatment methods can be divided into two basic categories: (1) Physio-chemical (as described above); and, (2) Biological (see below).

Physio-chemical pretreatments encompass all of the operations described above, including: grinding, milling, high-pressure steam explosion, acid or alkaline washes, or combination of several of these. Grinding or milling alone is not an effective pretreatment. These are often the first steps in reducing particle size, increasing surface area, and increasing porosity of the biomass for the subsequent enzymatic processing. Soaking, rinsing, or pressure treatment with acid and/or alkaline solutions to generate a slurry typically follows the

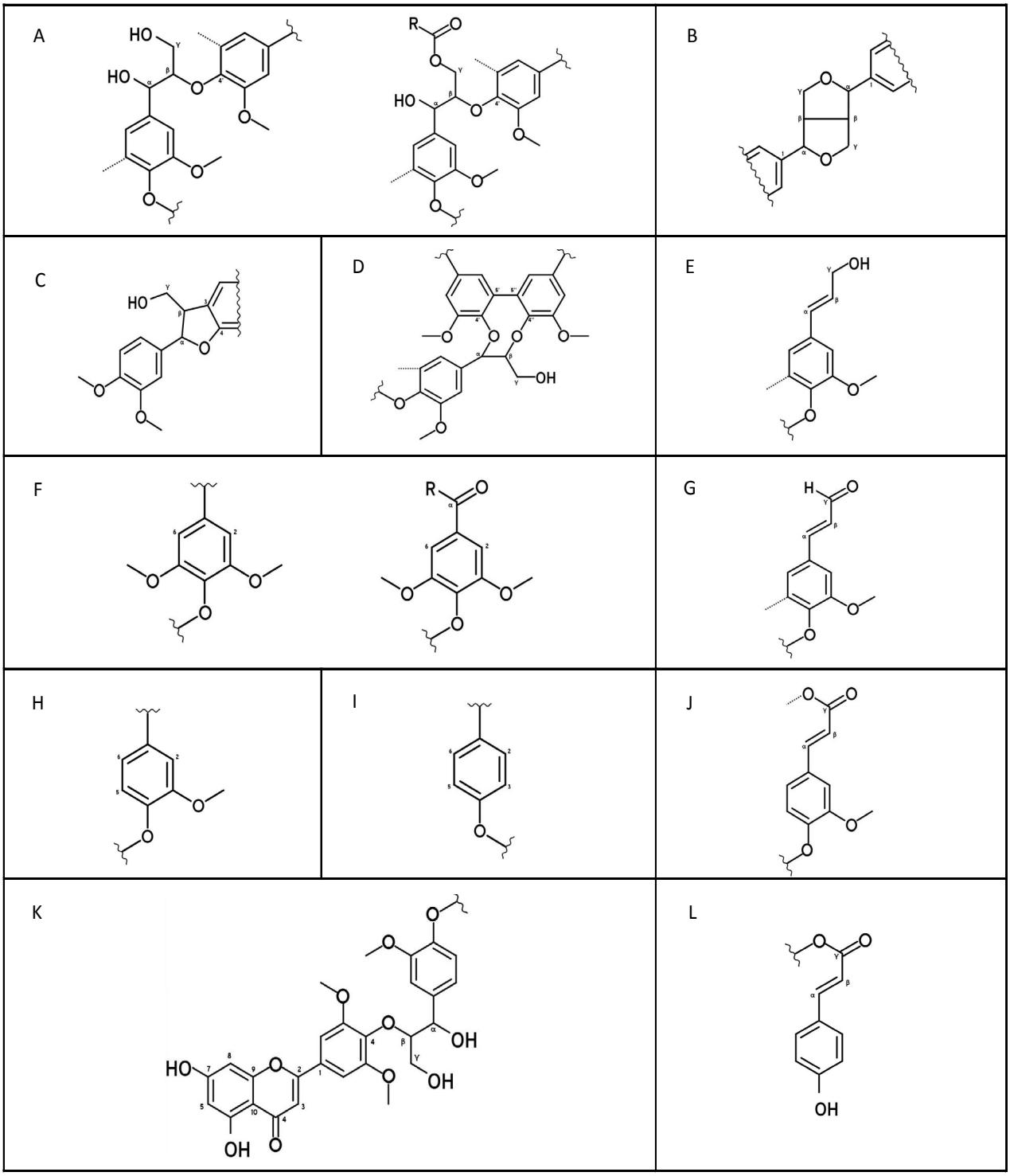


Figure 5: Main substructures present in the lignin fractions of bamboo (*D. brandisii*), as revealed as 2D HSQC NMR. (**A**) β-*O*-4' substructure (left) and β-*O*-4' substructures with acylated γ-OH (right); (**B**) resinol substructure formed by β-β' coupling; (**C**) phenylcoumaran substructure formed by β-5' coupling; (**D**) spirodienone substructure formed by β-1' coupling; (**E**) cinnamyl alcohol end-groups; (**F**) syringyl units (left) and oxidized syringyl units bearing a carbonyl at Cα (right); (**G**) cinnamyl aldehyde end-groups; (**H**) guaiacyl units; (**I**) *p*-hydroxyphenyl units; (**J**) ferulate units; (**K**) *p*-coumarate units; (**L**) a likely incorporation of tricin into the lignin polymer through a G-type β-*O*-4' linkage redrawn from [40].

aforementioned mechanical disruption processes [27]. Acid washes are often conducted at elevated temperature (130-210°C) with dilute acids (*see* DAL above) to solubilize 80-100% of the hemicellulose fraction primarily into monomers, liberating cellulose without significantly disrupting the crystalline structure of the target polysacchearide [36]. Although relatively efficient, acid pretreatment has drawbacks. Specifically, the neutralization of acid, equipment corrosion, and the necessary removal of contaminant compounds (e.g., lignin) that are

inhibitory to subsequent enzymatic hydrolysis and yeast fermentation stages contribute significantly to the overall cost of the ethanol production process [107,108].

Alkaline pretreatment is performed at room temperature and ambient pressures [26]. Alkaline washes can solubilize lignin thereby releasing hemicellulose and causing swelling of the cellulose structure. This latter phenomenon results in reduced cellulose crystallinity expanding the surface area of the cellulose structure thus increasing enzyme access to this targeted polysaccharide [107]. In other words, the cellulose structure becomes more amorphous and more susceptible to enzymatic attack. Since high temperatures and pressurized treatment is not required when applying alkaline washes, this type of pretreatment can be more cost efficient from an energy-use perspective. More importantly, whereas acids result in the mineralization of lignin, alkaline treatments result in soluble lignin making separation of lignin more efficient [26,109].

Ammonia fiber explosion (AFEX), which leads to AXL (Table 2), is a type of alkaline treatment that is not conducted at room temperature and ambient pressure. AFEX uses liquid ammonia at elevated temperatures 60-100°C and pressures of 250-300 psi to "shock" the matrix for ~5 minutes, after which there is a rapid decrease in batch pressure, which expands the ammonia gas. This process is highly effective at hydrolyzing hemicellulose and reducing cellulose crystallinity [110]. What is unique about this type of pretreatment is that the relatively low temperatures do not produce chemical inhibitors to downstream processes [27]. Furthermore, there is not a lot of chemical waste since nearly 100% of the ammonia can be recovered. The drawback of AFEX is that it does require elevated pressures and thus higher energy usage. Moreover, AFEX results in a mineralized lignin residual, making it difficult to extract lignin from the polysaccharide fractions [30].

High-pressure steam explosion, which produces SEL, is similar to AFEX. However, instead of using ammonia, water is used. High-pressure steam explosion at 102-143 psi and temperatures of 160-240°C is followed by a sudden reduction in pressure. This expands the plant fibers (as in AFEX); however, it does not significantly alter cellulose crystallinity. High-pressure steam explosion hydrolyses 80-100% of the hemicellulose component [36]. It does not solubilize the lignin component of the matrix; however, it does modify lignin (Table 2) [57]. The absence of acids or alkaline solutions in steam explosion pretreatment renders it "environmentally-safe" and it is the preferred methods of many operating cellulosic facilities [28]. Although steam explosion is quite efficient and overall environment-friendly, it does result in the production of acetic acid as well as furfural and phenolic compounds, which inhibit downstream biological processes [30].

Organosolvation, or Organosolv pretreatment, which results in OSL, is also conducted at elevated temperatures (150-200°C). However, instead of using ammonia or water, organic solvents such as methanol, ethanol, or acetone are employed. Organosolv results in a fairly soluble lignin residual (70%) and the generation of a relatively pure lignin co-product with minimum cellulose loss (<2%). Like steam explosion, however, biological inhibitors are also produced [107]. Furthermore, the use of elevated temperatures and the organic solvents result in added production costs [107].

Although there are multiple other pretreatment processes, dilute acid washes, alkaline washes, AFEX, high-temperature steam explosion, and Organosolv are among the most widely used. Most feedstock is subjected to some type of mechanical process prior to application of these physio-chemical pretreatment processes. Although each of these has its advantages, noted disadvantages are also part of the pretreatment selection process and contribute to overall production costs. With the exception of steam explosion, these processes also produce hazardous wastes or require recovery of environmentally hazardous materials. To reduce costs and to minimize the handling of hazardous materials, biological pretreatments have also been studied, and in some cases employed, as a substitute or for use in conjunction with physio-chemical pretreatments. Biological pretreatments may be either abiotic (i.e., the use of enzymes) or biotic (i.e., microbial-based). Several reviews on the use of naturally-derived as well as engineered enzymes for lignocellulose deconstruction have been published [111-113]. In fact, there are companies with the sole purpose of designing and developing enzymes for bioethanol production processes. Thus, only a brief overview of abiotic treatments is provided here.

Abiotic (Enzyme-mediated) lignocellulose deconstruction

To breakdown lignocellulose via abiotic deconstruction processes, four main classes of enzymes must be used: (1) endoglucanases; (2) exoglucanases; (3) β-glucosidases; and, (4) cellobiose dehydrogenases. Enzymes may be natural or engineered. They may be applied individually in sequential steps or as part of enzyme "cocktails" (i.e., mixtures). They may be delivered free in solution or bound to scaffolds. Other specialized enzymes beyond these four core classes may also be used to enhance lignocellulosic matrix deconstruction.

Endoglucanases (a.k.a., endocellulases) are glycosyl hydrolases that act on the interior regions of cellulose molecules. Endocellulases are most effective at cleaving amorphous cellulose strands. (This alone justifies the use of pretreatment technologies that expand the matrix and reduce crystallinity). Endoglucanase activity generates new free ends by cleaving internal cellulose bonds [36], further decreasing cellulose crystallinity and providing substrate for exoglucanases (see below). Optimized deconstruction using endocellulases results in a robust release of cellobiose, a disaccharide composed of two β(1,4)-linked glucose molecules. Subsequent breakdown of cellobiose releases the desired glucose substrate for yeast-mediated fermentation [36,114].

Exoglucanases (a.k.a., exocellulases or cellobiohydrolases) act on the free ends of cellulose molecules. There are two general subtypes of cellobiohydrolases (CBH): CBHI and CBHII. CBHIs bind the reducing end of cellulose cleaving two residues in from the end to release a cellobiose molecule [115]. CBHIIs bind the non-reducing ends also cleaving two residues in and releasing cellobiose [115]. CBHs are generally processive enzymes making several sequential cuts in a "ratcheting" manner before dissociating from the cellulose substrate. β-glucosidases act to breakdown cellobiose into glucose; however, β-glucosidases also readily digest short amorphous cello-oligosaccharides [36].

Cellobiose dehydrogenases are enzymes that oxidize cellobiose [116]. Unlike other cellulases, which act via hydrolysis, cellobiose dehydrogenases produce H_2O_2 and oxidize cellobiose to reduce Fe^{3+} to Fe^{2+} or Cu^{2+} to Cu^{+} [33]. Then, reactions between H_2O_2 and the reduced metals produce hydroxyl radicals facilitating the depolymerization of cellulose, hemicellulose, and lignin [33,117-119]. CDHs also reduce phenol radicals preventing the association of lignin monomers and inhibiting polymerization of lignin [116,119,120]. CDHs also reduce quinones to hydroxylated compounds thus preventing the repolymerization of quinones into lignin [120]. Apart from these primary functions, it is suggested that CDH can modify lignin further by cleaving β-ether bonds, demethoxylating aromatic structures, and forming hydroxyl groups [121]. Interestingly, quinones reduced by CDHs are susceptible to phenol-oxidizing enzymes such as lignin

peroxidase, which, if present, further aids depolymerization [120].

In fact, there are many enzymes apart from these basic endoglucanases, exoglucanases, β-glucosidases, and cellobiose dehydrogenases that could enhance the deconstruction of lignocellulosic biomass. Although developing a single cocktail composed of all of these different classes of enzymes may sound appealing, enzyme-mediated processes are synergistic. In some cases, simultaneous introduction of multiple enzymes will actually reduce (or completely inhibit) the activity of a given enzyme within the cocktail.

Developing an optimal enzyme-mediated pretreatment often involves developing multiple cocktails that are delivered sequentially. Understanding enzymatic action within the lignocellulosic matrix is essential. For example, products of enzymatic activity can act as inhibitors to the very enzymes that generated them. Cellobiose inhibits the activity of endoglucanases [122] and cellobiohydrolases [36]. Similarly, β-glucosidases are inhibited by their product, glucose [36]. Engineered lignocellulose deconstruction enzymes are often developed to increase catalytic activity and viability under harsh industrial conditions such as high-temperatures or extremes of pH [113]. As enzyme technology advances, compatibility of enzyme classes for use within cocktails has become a research focus [9,123]. Moreover, delivery strategy has become an intense area of investigation. In contrast, to having enzymes free in solution, enzyme immobilization platforms [124,125] and mobile enzyme sequestration platforms [9,126] are being studied as alternative ways to optimized the catalytic activity of multi-enzyme systems. The exploration of multi-enzyme systems for optimizing lignocellulose biomass deconstruction does not appear to be waning. Both bio-prospecting and engineering of enzymes has led to the development of enzymes that can resist temperatures to 90°C and pH<4 [102,113]. These exciting developments further support the reconsideration of previously under-studied SGB feedstocks for bioethanol production. As enzyme technology improves, more high-yield feedstock options may become available. Interestingly, some of the most effective enzymes used in industry were derived from microorganisms – specifically, bacteria and fungi. Although the scientific community and industry have excelled in developing abiotic enzyme options for lignocellulose deconstruction, it can be argued that microorganisms still hold the best secrets to deconstructing lignocellulose using multiple enzymes. Understanding how microorganisms breakdown lignocellulose or, even, using active cultures as pretreatment is an intense area of ongoing research.

Non-standard specialty enzymes from fungi

Although lignin-degrading enzymes are not an industrial standard in the production of biofuels, ligninolytic enzymes are worth mentioning due to their natural occurrence in model microorganisms and their potential to enhance current pretreatment technologies [29,127]. Perhaps the four most promising lignolytic (a.k.a., ligninolytic) enzymes for enzyme-mediated pretreatments are (Table 5): Lignin Peroxidases (LiP), Manganese Peroxidases (MnP), Versatile Peroxidases (VP), and Laccases (Lac).

LiP is a heme peroxidase (i.e., a Fe^{2+} - containing enzyme that catalyzes oxidation by hydrogen peroxide). It has a high redox potential and performs well at low pH with optimal catalytic efficiency at pH 3 [127-129]. LiP catalyzes the oxidation of non-phenolic methoxylated aromatic rings [128-130] generating cation radicals that induce a variety of reactions, including: the cleavage of C_α–C_β bonds, cleavage of aromatic ring structures, β-O-4 cleavage, and the formation of aldehydes and/or ketones [119,127,129]. LiP performs single-electron oxidations resulting in two distinct conformational states [15,119,131]. These states are LiPI and LiPII. LiP contains Fe^{3+} in its heme domain (Table 5), which is oxidized by H_2O_2 to produce LiPI with Fe^{4+}. LiPI then oxidizes a substrate (e.g., a quinone) resulting in LiPII and a substrate radical. LiPII then oxidizes a second substrate producing another substrate radical returning LiP to its original state with Fe^{3+} (and no radical). In the presence of excess H_2O_2 (or absence of reducing substrate), LiPII reacts with H_2O_2 to produce a relatively inactive ferric-superoxo complex ($Fe^{3+}O_2^-$) designated as LiPIII [119,132].

The addition of veratryl alcohol has been shown to revert the inactive LiPIII to a native LiP state by displacing O_2^-. Veratryl alcohol prevents the formation of LiPIII by reverting LiPII back to LiP [128-132]. Although LiP can oxidize phenolic compounds to a limited extent, these reactions result in the accumulation of the inactive LiPIII since phenoxy radicals are unable to convert LiPIII to its native state [119].

MnP is a high redox potential heme peroxidase that also exhibits multiple conformational states – namely, MnPI, MnPII and MnPIII [133]. If H_2O_2 is present the enzyme converts to the MnPI state. MnPI is reduced resulting in a conformational change to the MnPII state and the production of Mn^{3+}. Another manganese reduction restores the enzyme to its native state releasing water and another Mn^{3+}. Similar to LiP, in the presence of excess H_2O_2 (or absence of reducing substrate), MnPII forms an inactive $Fe^{3+}O_2^-$ state, designated MnPIII. However, the inactivation is not as stable as seen in LiPIII since the superoxide of the inactive MnPIII can lose an electron to Mn^{3+} reverting the enzyme to its native state [119]. Mn^{3+} is a highly oxidative cation that cleaves phenolic compounds, particularly syringyl and vinyl substituted side-chains [29]. It has also been demonstrated that Mn^{3+} readily cleaves alkyl-phenyl and C_α–C_β bonds and methylates aromatic ring structures [127,129]. Products resulting from Mn^{3+} activity include small molecular weight compounds such as oxalic acids, peroxyl radicals, and acyl radicals, which are capable of diffusing into the cell wall and disrupting lignin from inside, while bulky enzymes are restricted to the outer surface of the cell wall [29].

VP is another peroxidase that is a hybrid of LiP and MnP capable of cleaving phenolic and non-phenolic compounds independent of the presence of Mn^{2+} [15,29].

Lac is a copper-containing oxidase that typically oxidizes aromatic amines and phenols by single-electron oxidations coupled to the four-electron reduction of O_2 to water [134]. The oxidation of aromatic compounds can result in: phenoxy radicals that randomly oxidize C_α-hydroxyls to ketones; cleaved alkyl-aryl bonds; demethoxylation of methoxy-substituted aromatics; or, cleaved C_α-C_β bonds. Such reactions can lead to lignin polymerization (a precursor to lignin depolymerization), while others modify byproducts of lignin degradation [15,135]. In the presence of ABTS (2,2'-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt), laccase has also been observed to oxidize non-phenolic compounds [15,127]. Although this is not a complete list of lignolytic enzymes with potential applications in industrial lignocellulose deconstruction processes, these lignin-modifying enzymes provide an example of the diverse array of enzymes that can be derived from natural sources to refine current or develop new pretreatment strategies. Interestingly, all of these enzymes are derived from living organisms-mainly microorganisms such as bacteria and fungi. Thus, the question begs, if natural systems have evolved the best repertoire of enzymes to break down cellulosic biomass, then why not simply use these living microorganisms instead of spending large amounts of time and great sums of money trying to replicate *in vitro* (via protein over-expression, and protein engineering) what these microbes do naturally?

Enzyme	Substrate(s)	Cofactor(s)	Product(s)	Mechanism§	Refs
LiP	non-phenolic methoxylated aromatic rings	veratryl alcohol; H_2O_2	cation radicals; aldehydes; ketones	$LiP(Fe^{3+}) + H_2O_2 \rightarrow \cdot LiPI(Fe^{4+}) + H_2O$ $\cdot LiPI(Fe^{4+}) + R \rightarrow LiPII(Fe^{4+}) + \cdot R$ $LiPII(Fe^{4+}) + R \rightarrow LiP(Fe^{3+}) + \cdot R$	[20] [119] [133]
MnP	Mn^{2+}	H_2O_2	phenoxyl radicals; Mn^{3+}	$MnP + H_2O_2 \rightarrow \cdot MnPI(Fe^{4+})$ $\cdot MnPI(Fe^{4+}) + Mn^{2+} \rightarrow MnPII(Fe^{4+}) + Mn^{3+}$ $MnPII(Fe^{4+}) + Mn^{2+} \rightarrow MnP + Mn^{3+} + H_2O$	[20] [133]
Lac	phenolic compounds; aromatic amines	O_2	phenoxy radicals; methanol; ring cleavage; ketones	$Lac\ (4\ Cu^{2+}) + 4R \rightarrow Lac(Cu^{+}) + 4\ \cdot R + 4H^{+}$ $Lac\ (Cu^{+}) + O_2 \rightarrow Lac\ (2\ Cu^{2+};\ 2\ Cu^{+})$ $Lac\ (2Cu^{2+};\ 2Cu^{+}) + 2H^{+} \rightarrow Lac\ (4Cu^{2+}) + H_2O$ $Lac\ (4\ Cu^{2+}) + 2H^{+} \rightarrow Lac\ (4Cu^{2+}) + H_2O$	[20] [119] [133]
VP	phenolic and non-phenolic compounds; hydroquinone substituted phenols	H_2O_2	phenoxy radicals; methanol; ring cleavage; ketones	$VP(Fe^{3+}) + H_2O_2 \rightarrow VPI(\cdot O—Fe^{4+}) + H_2O$ $VPI(\cdot O—Fe^{4+}) + R \rightarrow VPII(O—Fe^{4+}) + \cdot R$ $VPII(O—Fe^{4+}) + R \rightarrow VP(Fe^{3+}) + H_2O + \cdot R$	[119] [133] [189]

Table 5: Chemistry of Lignin Degrading Enzymes in Fungi. R - substrate; - radical (□R - substrate radical); $LiP(Fe^{3+})$ – LiP with Fe^{3+} in the heme domain (notation follows suit for other enzymes).

Biological (Microbial-based) lignocellulose deconstruction

Even though enzyme overexpression and enzyme engineering can be costly and time-consuming, these abiotic biological approaches to degrade lignocellulosic biomass offer some level of control in the design and development of pretreatment processes. In contrast, biotic pretreatments, which use viable microbes to initiate lignocellulose deconstruction are not as tractable. Maintaining live cultures under optimal growth conditions is challenging. Living organisms can undergo sudden life cycle changes in response to subtle environmental cues, adapt, and turn-on/off protein expression without an identifiable reason. Nonetheless, the idea of using bacteria or fungi to pretreat biomass is decades old. Although some paper and pulp operations do use microbial-based pretreatments for woody biomass, few for-profit bioethanol facilities employ this strategy. Still, microbial-based treatment has promise. Several studies have focused on use of the thermophilic anaerobic bacterium *Clostridium thermocellum* [136] and soft-rot fungus *Trichoderma reesei* [137,138]. Indeed, it is from these two microorganisms that many of the aforementioned enzymes were derived.

Bacterial pretreatments have largely focused on thermophilic anaerobic ethanologenic bacteria (including C. thermocellum), which are capable of producing an array of high-specificity cellulases and hemicellulases to depolymerize holocellulose into monosaccharides, which the bacteria then use to ferment into ethanol [139]. This direct route to fermentation is attractive; however, there are multiple drawbacks in relying on bacterial cultures (especially anaerobic cultures), as the sole process for taking raw lignocellulosic biomass all the way through to the ethanol production stage.

Notably, anaerobic bacteria do not penetrate into the cellulosic matrix but instead produce cellulosomes - protein scaffolds that attach multiple enzymes to the bacterial cell wall [139]. Although bound to the bacterial cell wall, cellulosomes demonstrate enough flexibility to tightly bind to microcrystalline cellulose. The close proximity of the scaffoldin-bound enzymes results in significant enzymatic synergy that adds to greater cellulolytic capability than most engineered enzyme cocktails [14,36,139]. Attempts to mimic natural cellulosomes by engineering artificial cellulosomes or "mini-cellulosomes" have met with limited success. Immobilizing platforms allow for sufficient control of enzyme composition [125]; however, access to substrate is limited since platform-bound enzymes are unable to diffuse into the slurry. More recently, mobile enzyme sequestration platforms (mESPs), which overcome this limitation, have been developed [9,126]. Another advantage of mESPs is that multiple enzyme classes may be bound to a single platform or multiple platforms with different enzyme complements may be used to diffuse into the slurry to access substrate. However, this advantage also presents a major challenge. With either the immobilized platforms or mobile platforms, finding the most catalytically efficient enzyme complement is difficult. Via evolutionary processes, natural systems have developed to express highly synergistic enzyme complements with substitutions occurring in response to changing environmental conditions. Most natural cellulosome scaffolds employ 5-9 enzymes at a time. The ability to emulate these dynamic natural systems, or to even develop more advanced platform systems that may harbor 10-18 enzymes per scaffold at a time, makes optimizing artificial platform systems extremely difficult. As a simple example, the binding of both exoglucanases and endoglucanases to the same mESP actually decreases the activity of each individual enzyme class [102]. This is likely due to a "tug-of-war" at the molecular level whereby the exoglucanase seeks to bind a reducing or non-reducing end while the endoglucanase is drawn towards interior points of the cellulose chain. Mobile platforms may be the technology of the future; however, natural microbial systems are still in the lead when it comes to cellulosome design.

Aerobic bacteria (e.g., species of the genus *Streptomyces* of the cellulolytic class Actinomycetales) do not produce cellulosomes like anaerobes. Aerobic bacteria are similar to lignocellulolytic fungi in that both types of microbes produce and secrete free enzymes extracellularly. By positioning themselves in the immediate vicinity of the lignocellulosic substrate, enzymatic diffusion is minimized (eliminating the need for a cellulosome) and the cell wall may be penetrated [139].

Advantages of bacterial pretreatments include their unique cellulolytic and hemicellulolytic systems as well as their ability to directly ferment a variety of monosaccharides into ethanol. In the case of thermophilic bacterial strains (i.e., those with optimal growth above 60°C), microbial-based pretreatments could reduce costs associated with lowering bioreactor temperatures prior to the addition of bacteria and prevent contamination [139]. The disadvantages of anaerobic bacteria are requirements for strictly anaerobic conditions. Anaerobes also exhibit low metabolisms resulting in slow enzyme production and growth rates. Thermophilic bacteria (aerobic and anaerobic) generally have low-tolerance to the ethanol produced as well as other biological inhibitors that may remain from preceding physio-chemical pretreatments [14,139]. Interestingly, thermophilicity and anaerobic

metabolisms are generally coincident characteristics in bacteria and only two species of aerobic thermophilic cellulolytic bacteria have been well-characterized [36].

Biological pretreatment of lignocellulosic biomass with fungi has also been studied as a way to reduce crystallinity and modify cellulose to a more amorphous state. Depending upon the species, primary targets for digestion biomass may be the lignin component or the holocellulose [15]. Fungi are generally aerobic with faster metabolisms than those of the thermophilic anaerobic bacteria. Therefore, they receive more attention for commercial cellulase production [14]. Four fungal species (*Neurospora crassa*, *Fusarium oxysporum*, *Paecilomyces lilacinus*, and *Mucor indicus*) as well as the brown-rot fungus, *Neolentinus lepideus*, are known to convert xylose directly into ethanol [140]. A highly efficient xylose-fermenting strain, *Trametes versicolor* (strain KT9427), was isolated from fruiting bodies on wood decay and shown to produce 0.44 g EtOH/g xylose consumed. *T. versicolor* was also able to produce ethanol directly from unpreatreated lignocellulosic feedstocks. Although the ethanol yield was only 9.8 g/L after 96 hours, extended fermentation times and/or pretreatment may push the yield closer to levels needed for commercial use [141].

White-rot (WRF), brown-rot (BRF), and soft-rot (SRF) fungi are among the most efficient degraders of lignocellulose [29]. Although most fungi are feedstock-specific [36], several well-studied white-rot fungi, including: *P. chrysosporium, P. ostreratus,* and *T. versicolor* - are successful lignin degraders across multiple feedstocks varying from bamboo to corn stover and pine [2,15,29,142]. Unlike WRF, which can either be selective to lignin or deconstruct both holocellulose and lignin equally, BRF and SRF typically degrade holocellulose while only inducing minor chemical modifications to lignin [19,29,119]. Although it has been suggested that SRF can mineralize lignin, rigorous experimentation has not been conducted to support this claim [14,127].

The majority of the research on the isolation of industrial fungal cellulases has focused on the SRF *Trichoderma reesei* due to its ability to produce high yields of extracellular cellulases (up to 50 g/L) [119]. *T. reesei* has also been utilized to over-produce genetically inserted laccase genes which can be used to breakdown lignin. Yields up to 920 mg Lac/L are possible [119]. This yield is considered to be very high and *T. reesei* has become a model system in enzyme production. The success of *T. reesei* has prompted extended investigation of WRF species as candidates for industrial microbial-based pretreatments for the degradation of lignocellulosic biomass in ethanol production. As a consequence, BRF have been largely overlooked [14,15,107].

Justifiably, fungi are considered promising candidates for microbial-based biological pretreatment. Advantages of fungal pretreatment include mild process conditions, the potential for efficient lignin degradation (or conversion of lignin into value-added by-products), low energy requirements, and minimal hazardous product formation. Fungi also prefer low-pH environments and grow well at elevated temperatures (45-50°C), thereby reducing the energy input required to neutralize batch reactors [143].

The fungi: specialists on lignocellulose

There are three main types of fungi, categorized based on differences in metabolism. SRF are typically from the phylum Ascomycota. SRF readily degrade polysaccharides and are able to oxidize some syringyl-based lignin (but not guaiacyl-derived lignin). Thus, SRF-mediated degradation is largely limited to regions rich in S-type lignin while G-type lignin-rich regions are left largely intact [20].

Due to limited lignolytic action, SRF-degraded wood becomes soft, but not stringy [119]. From an industrial use perspective, it should be noted that SRF generally require high nitrogen levels to effectively degrade wood and they are found naturally in moist or aquatic habitats [119].

WRF are generally from the phylum Basidiomycota. Unlike SRF and BRF, WRF have evolved a secondary metabolism for lignin and specialize in deteriorating hardwoods and other high-lignin content species. Interestingly, in some WRF species, carbon and/or nitrogen limitation will stimulate lignolytic activity [14]. Although no known fungus is capable of surviving on lignin alone, lignin provides an additional energy resource under nutrient-depleted conditions [144]. In contrast to BRF, WRF mediate cell degradation without regard for the degree of lignification in microdomains of the cell wall structure [20]. Depending upon the species, some WRF exhibit "lignin-selectivity"; in other words, they will oxidize lignin releasing CO_2 and water at a higher rate than they degrade polysaccharide [2,15,119,145]. It should be noted that WRF only depolymerize the cell wall to the extent needed for baseline fungal metabolism [20]. In advanced stages of white-rot decay, protolignin is completely mineralized leaving white stringy polysaccharides – hence, the name "white-rot" [2,146]. *P. chrysosporium* is arguably the best-studied WRF [2,146].

Similar to WRF, BRF are generally Basidiomycota (Figure 6). In fact, studies demonstrate that many WRF and BRF are very closely-related [20]. Phylogenetic studies indicate that some species of WRF and BRF share the same genus, while others suggest that some BRF species evolved from WRF [127,144,147]. Seemingly arbitrary deletions of genes for select lignin-degrading enzymes in BRF, which are otherwise genetically identical to a closely-related WRF, support this latter idea. It is argued that by evolving a metabolism capable of bypassing lignin degradation and acting directly on polysaccharides, BRF are the more evolutionarily advanced species [144,148].

Regardless of the evolutionary relatedness of WRF and BRF, there are key physiological distinctions. Unlike WRF, BRF continue to degrade polysaccharides in excess of their ability to metabolize the products [12,20]. BRF generally metabolize softwood holocellulose with minimal deterioration of lignin structures [127]. Although some demethylation and partial oxidization of lignin is consistently reported, BRF do not appear to significantly modify aromatic ring structure [13,119,149]. BRF generally lack high-potential class II peroxidases (POD) including LiP, MnP and VP [150]. However, a few studies indicate that some BRF can produce select PODs [151]. Conversely, *Schizophyllum commune*, a WRF, appears to lack PODs [150]. MnP and LiP activity was observed in BRF *Laetiporus sulphureus* [152]. LiP activity is reported for BRF *Polyporus ostreiformis* [153] and both Mn-dependent and Mn-independent peroxidase activity (and laccase activity) is reported for BRF *Fomitopsis pinicola* [154]. Different genetic profiles between WRF and BRF with respect to expressing different enzymes support distinct pathways to degrading lignocellulose. For example, laccase activity is reported in a number of BRF including: *Gloeophyllum trabeum*, *Coniophora puteana*, *Wolfiporia cocos*, and *Postia placenta* [134,144,155,156]. Although *P. placenta* is closely related to the well-studied WRF *Phanerochaete chrysosporium*, *P. chrysosporium* does not produce laccases suggesting that laccases may not be essential for lignin degradation and may play a more important role in the production of radicals when expressed by the BRF [144]. The nature of BRF infection cycles on woody biomass may provide added insights into the core functions of enzymes expressed in these fungi.

During initial infection, brown-rot hyphae enter the cell through pits in the membrane, which also serve as a potential pathway to

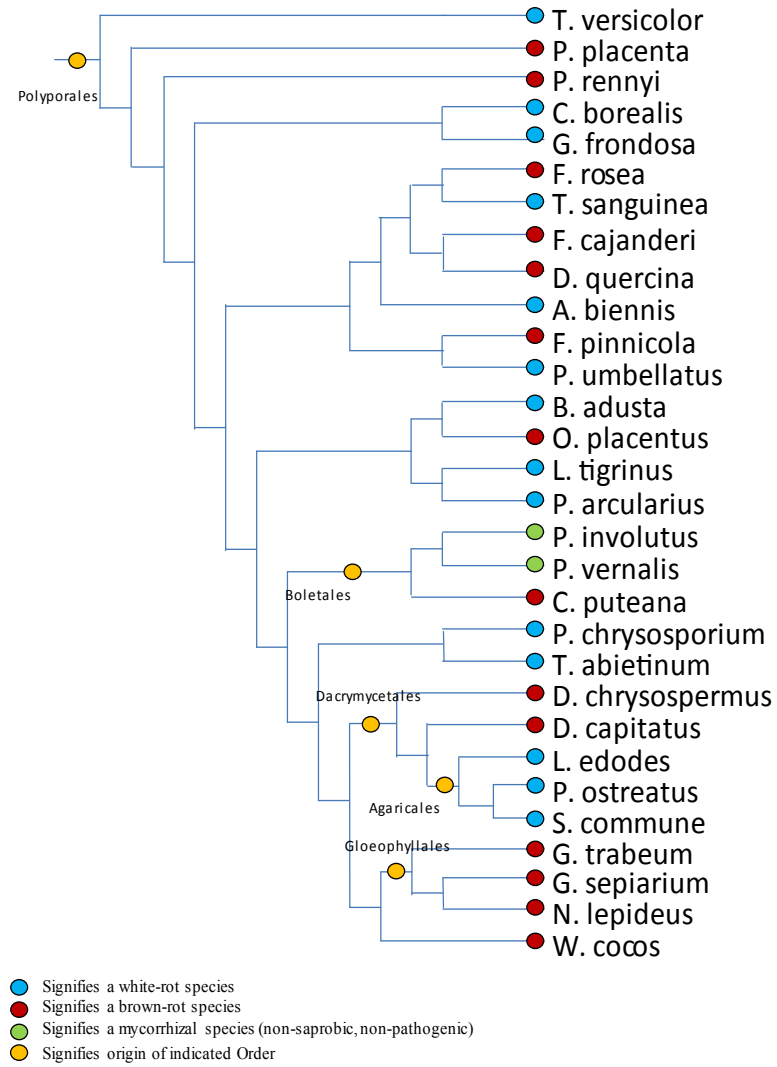


Figure 6: Phylogenetic relationships within the Class Agaricomycete (based on 28s and 5.8s rRNA analysis).

penetrate neighboring cells [19,157]. Once established in the cell lumen, the hyphae secrete a glucan layer, which adheres hyphae to cell wall [19,158]. High lignin content microdomains and the large molecular masses of cellulolytic enzymes, prevent the direct enzymatic breakdown of lignin [12]. However, low molecular weight radicals are released that are able to penetrate dense microdomains (e.g., S3 layer) to deteriorate polysachharides and modify lignin from inside [19]. The progression of brown-rot infection results in a higher rate of hemicellulose breakdown than cellulose [12,13]. Ultimately, all polysaccharides may be degraded leaving brown crumbly residues of modified lignin - hence the name "brown-rot" fungi. *Gloeophyllum trabeum* is perhaps the best-studied brown-rot species in this respect [20,150].

The general absence of lignin degrading enzymes (at least during the initial decay phase) in BRF may shift the burden of decay activity onto low-molecular weight compounds and radicals that penetrate through lignin-dense microdomain so that access to and the breakdown of holocellulose may occur. Two examples of such low-molecular weight compounds are 2,5-dimethoxyhydroquinone (2,5-DMHQ) and oxalic acid, which are both produced by *P. placenta* [156]. 2,5-DMHQ reduces Fe^{3+} for the production of H_2O_2, which generates hydroxyl radicals via the Fenton reaction (described below).

Oxalic acid will acidify wood to pH 1.5-4 [117,156-159], optimally activating hydrolytic enzymes (e.g., xylanases) and facilitating acid hydrolysis of hemicellulose [159,160]. Oxalic acid is also reported to chelate iron bound to wood, providing a ready supply of iron for Fenton chemistry [161,162]. However, it is also important to note that the Fenton reaction is inhibited at pH<4 by the formation of a stable iron-oxalate complex, which serves to protect fungal hyphae from oxidative damage associated with H_2O_2 production [19,117]. Beyond these examples, BRF reliance on radical producing enzymes and low-molecular weight compounds to degrade lignocellulose is further supported by the fact that most BRF possess few genes that encode cellulases (e.g., exoglucanases). For example, many of the better-studied BRF species lack CBH genes as well as the genes encoding for the cellulose binding modules (CBM) that facilitate binding of CBHs to cellulose [144,150].

Although *G. trabeum, C. puteana*, and *S. lacrymans* have at least one copy, the majority of BRF also lack cellobiose dehydrogenases [150,151]. Interestingly, *P. placenta* appears to have no exocellulases, no cellobiose dehydrogenases, and only two putative β-1-4-endoglucanases [144] with a later report indicating that *P. placenta*

encodes five β-1-4-endoglucanases [151]. Despite the roughly equivalent expression of hemicellulases and pectinases in WRF and BRF [150], the apparent dearth of exocellulases and cellobiose dehydrogenases in BRF support the suggestions that either: (a) other mechanisms are in place to degrade lignocellulose; or, (b) some of the 49% of proteins with unidentified functions in Basidiomycota may code for unknown cellulases that match or exceed the performance of white-rot cellulases [150]. Although the latter is possible, the former is more likely.

Specifically, it is more likely that BRF rely significantly on radicals and low-molecular weight oxidants for their decay activity. Indeed, radicals and low-molecular weight oxidants (LMWOs) are reported to be the main agents responsible for brown-rot decay. As mentioned above, even the smallest of cellulolytic enzymes are unable to penetrate lignin-rich microdomains to reach the polysaccharide rich regions. This drives selection in favor of small catalytic molecules. Several mechanisms producing such compounds have been described. The Fenton reaction, discovered in 1965 by Halliwell [163], is speculated to be the primary reaction responsible for brown-rot decay.

Fenton Reaction:

$$H_2O_2 + Fe^{2+} + H^+ \rightarrow HO^- + Fe^{3+} + H_2O$$

The only limitation of the Fenton reaction in decay is the lack of naturally occurring hydrogen peroxide in wood; however, the fungi have evolved several mechanisms to produce their own hydrogen peroxide.

The role of laccase in quinone-redox reactions has already been addressed in detail in earlier sections. CDH can also participate in the formation of hydroxyl radicals in the presence of iron and oxalic acid. Oxalic acid is a known chelator of Fe^{3+}. Chelated Fe^{3+} can be reduced by CDH to Fe^{2+}-monooxalate or uncomplexed Fe^{2+} at a pH<2.5. Both complexed and uncomplexed Fe^{2+} are stable at pH 2.5 and diffuse away from hyphae to regions with lower levels of oxalic acid. In these higher pH regions it reacts with oxalic acid to form Fe^{2+}-dioxalate. Diffusing to regions of pH ~4, Fe^{2+}-dioxalate autoxidizes to produce $HOO^{\cdot}$ which is reduced by Fe^{2+} to produce H_2O_2 [33,117]. The diffusion component of the mechanism is especially important in the protection of hyphae from the oxidative activity of H_2O_2, which can be toxic to the fungus. In addition, CDH has also been reported to produce phenol radicals that reduce Fe^{3+} to Fe^{2+} and produce hydroxyl radicals in the presence of H_2O_2.

Interestingly, *G. trabeum,* a notable producer of 2,5-DMHQ, contains very little oxalic acid within the vicinity of its hyphae [159]. However, *G. trabeum* produces oxalate decarboxylase, which converts oxalate into formate and CO_2, suggesting fungal regulation of oxalic acid [159]. Oxalate decarboxylase is common expressed in WRF to maintain stable concentrations of oxalic acid, which, at high concentrations, inhibits lignolytic peroxidases. Other enzymes including alcohol oxidase isolated from *G. trabeum* and *P. placenta* are also capable of producing hydrogen peroxide [144,164]. Demethylation of lignin can produce methanol, which is toxic to fungi. Both WRF and BRF are capable of demethylating lignin and appear to efficiently express alcohol oxidases [19], which have a high affinity for methanol [164]. In the presence of alcohol oxidases, methanol is converted to formaldehyde generating H_2O_2 in the process [164]. Glyoxal oxidase (GLOX) is another common H_2O_2 producing enzyme which functions by catalyzing the oxidation of aldehydes to carboxylic acids concomitantly reducing molecular oxygen to hydrogen peroxide by the following reaction [165]:

GLOX: $RCHO + O_2 + H_2O \rightarrow RCO_2H + H_2O_2$

These represent only a few of the mechanisms that have been characterized through which fungi produce H_2O_2. These mechanisms provide yet another example of the diverse ways in which fungi have evolved to support metabolisms focused on degrading lignocellulose. The genetic diversity of these fungi underlies the diverse proteomes (in terms of the enzymes they express), which in turn mediate the diverse strategies they use to deconstruct lignocellulosic biomass.

Bamboo fungi

A more environment-friendly pretreatment option ultimately may be the best approach for producing lignin co-products. Bamboo lignin decomposes across a broad temperature range but slowly [187], [188]. Bamboo biomass is more rapidly decomposed with the assistance of select fungi. The decay of bamboo by white-rot and brown-rot fungi has been well documented. Reports have cited as many as 60 different fungal taxa engaged in the natural decomposition of bamboo culms [189,190]. Present in the decomposition community were a host of ascomycetes, basidiomycetes, and anamorphic fungi.

As previously mentioned, white-rot fungi in particular have been touted in the literature for their ability to degrade woody biomass by breaking down lignin [191]. Pre-treatment of biomass by white-rot is noted to "easily" convert complex lignin into simpler structures under mild conditions with low energy consumption [14]. In one study, 34 isolates of white-rot fungi were tested as degradative pretreatment for Moso bamboo culms [18]. Of these, 11 strains were determined by FT-IR analysis to possess high lignin-degrading ability. *Echinodontium taxodii* 2538 and *Trametes versicolor* – in particular, showed high delignification capacity with 5.15-fold and 8.79-fold increases (over control). In another study, the white-rot fungi *Irpex lacteus* CD2 and *E. taxodii* 2538 were reported to have an exceptional ability to degrade lignin and breakdown both *Phyllostachys pubescens* and Moso [192]. Although white-rot fungi have received the most attention as potential biological pretreatment options for deconstructing lignocellulose in bamboo, they are also known to mineralize lignin during delignification leading to lignin structures that are not readily converted to added-value lignin co-products. More recent evidence suggests that brown-rot fungi may serve a potential role in bamboo pretreatment strategies [193-197]. Using FT-IR, it was shown that the brown-rot fungus *Gloeophyllum trabeum* could efficiently breakdown p-hydroxyphenyl units in the lignin found in the middle lamella of Moso bamboo [194]. In another study, using FT-IR data, it was determined that two brown-rot fungi - *Coniophora puteana* and *Poria placenta* - could remove lignin from bamboo lignocellulose with little or no modification to the protolignin [196]. This is consistent with another study, in which it was observed that white-rot fungus *P. chrysosporium* preferentially degraded lignin over hemicellulose and cellulose, while the brown-rot fungus, *G. trabeum*, preferred the hemicellulose fraction over cellulose and lignin. In the case, of the latter, it is reasonable to suggest that by selectively attacking hemicellulose, lignin may be released relatively intact from the lignocellulosic matrix [193].

A potential drawback in developing brown-rot fungi-based pretreatments that generate valuable lignin products for the deconstruction of bamboo is that some brown-rot fungi target cellulose and other long chain polysaccharides that are the primary extracts of interest. Exploration of fungal biodiversity and potentially genetic modification of brown-rot fungi may lead to a biological pretreatment option for utilizing bamboo as a principal feedstock whereby both cellulose and lignin are preserved during the substrate separation process, allowing for both primary products and co-products to be produced in an efficient, non-competing manner [193].

The potential for Brown-Rot as a SGB pretreatment

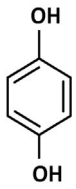
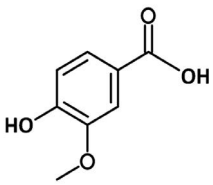
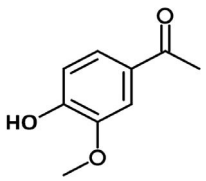
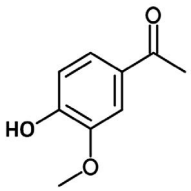
Despite the historical focus on WRF as *the* source for lignolytic enzymes and lignin degradation in lignocellulosic biomass, BRF, including *Postia placenta, Oligoporus fragilis,* and *Coniophora puteana*, have also been observed to express (and release) highly active lignolytic enzymes, including laccases [29,155,156,166] whereas the BRF *Polyporus ostreiformis* expresses both LiP and MnP [167]. Other brown-rots including *Laetiporus sulphureus, Wolfiporia cocos* and *Piptoporus betulinus* have been shown to express xylanases [122,168,169].

In short, there is overlap in the enzyme complements of SRF, WRF, and BRF. Whereas select BRF species can express the same classes of enzymes as WRF species, the converse does not hold, especially when degrading lignin. More thorough investigations of known BRF and characterization of novel BRF to further explore the nature of BRF proteome diversity may elucidate an even greater repertoire of enzyme subtypes that act on lignocellulose using novel mechanism. Indeed, it is this diversity within BRF proteomes and their unique metabolic strategies that have earned them recent attention as a potential pretreatment for various feedstocks. BRF pretreatments have been combined and compared to common physio-chemical pretreatments. Three BRF and one non-lignin-selective WRF (i.e., *Trametes versicolor*) were tested to determine their respective abilities to degrade steam-exploded pine [170]. The results of this study indicate that *T. versicolor, Antrodia xantha,* and *P. placenta* is all equally effective pretreatments for pine and more effective than *C. puteana*. After 6 weeks of fungal-based biological pretreatment on pine biomass. *C. puteana* resulted in a 12% increase in fermentable glucose while the other three fungal species produced a 17-23% increase in glucose.

This study also demonstrated that steam-exploded biomass is not toxic to these fungi. Arguably, with optimization, the yields may be even greater since these trials did not consider the potential for suboptimal compatibility between the fungus and selected feedstock. Fungal species-feedstock compatibility can play a significant role in determining yields. For example, another study demonstrated that high levels of corn stover decay could be achieved via biological pretreatment using the BRF *G. trabeum* but very little decay was observed when *P. placenta* was used [13]. Interestingly, BRF *G. trabeum* was also shown to release glucose from corn fiber (a co-product of corn-ethanol production) and as well as ferment the sugar produced to ethanol under anaerobic conditions [171]. This study demonstrates that it is not only compatibility of fungus and feedstock that is important but also the culture/environmental conditions under which pretreatment is conducted that can have significant impacts on outcomes. For practical use in an industrial setting, time-course of BRF decay is also an important parameter to consider.

C. puteana and *P. placenta* have been studied as potential microbial-based pretreatments for pine stock [12]. Using these BRF on Scots pine it was shown that >70% glucose recovery could achieve within 15 days of treatment. Using *G. trabeum* on aspen, another study demonstrated a 72% cellulose-to-glucose yield after 2 weeks of treatment [13]. Treatment of rice straw with LiP- and MnP-expressing BRF *P. ostreiformis* showed 18% lignin removal after 3 weeks [20].

It is clear that in addition to diversity across fungal species, there is significant diversity within the BRF such that certain feedstocks may be pretreated with select BRF under controlled conditions to produce high yields of desired products from both polysaccharides and lignin (Table 6). However, deconstruction pathway differences are not limited

Product	Known Producers	Structure	Reference
1,4-Dihydroxybenzene from benzyl alcohol	*T. palustris* (BRF) *G. trabeum* (BRF)		[186]
1,2,4-Trihydroxybenzene from 4- hydroxybenzoic acid	*T. palustris* (BRF) *G. trabeum* (BRF)		[186]
Vanillic acid from oxidation of guaiacyl units	Common to WRF and BRF expressing lignolytic enzymes		[20]
Vanillin from ferulic acid and vanillic acid	*P. cinnabarinus* (WRF)		[47] [187] [188]

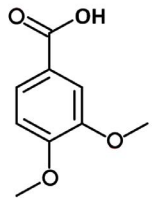
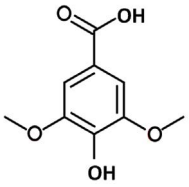
Veratric acid from oxidation of guaiacyl monomers of lignin and β-O-4 cleavage	Common to WRF and BRF expressing lignolytic enzymes		[20]
Syringic acid from oxidation of syringyl monomers of lignin	Common to WRF and BRF expressing lignolytic enzymes		[20]

Table 6: Lignin Degradation Products from Fungal Decay. Lignin structure and derived products after pretreatment with fungi and/or other lignin-modifying processes.

to between-species comparisons. There are reports that even under equivalent reaction conditions, different strains of the same species may execute different strategies to lignocellulose degradation. In one study [155], one of the four tested strains of *C. puteana*, strain COP20242, uniformly degraded all layers of the cell wall structure (regardless of lignin density), a characteristic typically attributed to white-rot decay. As mentioned previously, *C. puteana* was reported to produce laccase activity.

Interestingly, strain COP20242 was not the highest producer of laccase. Therefore another mechanism may be responsible for the enhanced lignin degradation. This provides an opportunity to explore the potential of BRF pretreatments through genetic modification of promising species in an effort drive select decay pathways that produce desired carbohydrate and lignin products.

Conclusions

In this paper, we have reviewed four separate bodies of literature focused on: (1) the role of lignin and lignin co-products in pulp/papermaking and bioethanol industries with detailed consideration of the distinct types of lignin structures that are produced from different lignocellulose treatment processes; (2) the biochemical composition and properties of second-generation biofuel feedstocks with emphasis on the potential use of alternative feedstocks, such as bamboo, as principal second-generation inputs; (3) pretreatment options for deconstructing lignocellulosic biomass with an overview of physio-chemical versus biological pretreatment strategies in both the bioethanol and pulp/papermaking industries; and, (4) the potential role of brown-rot fungi as a principal component for environment-friendly biological pretreatments of lignocellulosic biomass such that valuable lignin co-products may be produced to generate alternative revenue streams or otherwise offset production costs at bioethanol plants.

Although a comprehensive review of each of these topics is beyond the scope of any single paper, we have attempted to present information that lies at the intersection of these topics to provide an interdisciplinary perspective on the state of biofuels production worldwide. Cumulatively, the information and data provided here justify future efforts to examine potential gaps in current lignocellulosic biomass deconstruction and product development technologies. Further justification for continued research into novel feedstock and biological pretreatment regimens is supported by new interest in marketable modified lignin co-products as well as the ongoing characterization of novel microorganisms that may form the basis for innovative biotechnological breakthroughs.

It is clear that bio-prospecting and, perhaps, genetic modification of naturally isolated microorganism can only contribute to the effort of developing environmentally-safe biological pretreatments for bioethanol production. Microbial pretreatments are not energy intensive, they do not require the disposal or treatment of hazardous chemicals, and they are effective under optimal conditions. Challenges arise when attempting to scale-up proven processes from the lab to the industrial level. Despite often being feedstock-specific and requiring longer pretreatment times (usually 2-42 days) and the potential for live cultures to consume a portion of the desire polysaccharides for their own growth [15,36,119], microbial-based pretreatments should not be discounted. Reports on the use of cellulase-less fungal mutants that are limited to metabolizing the hemicellulose fraction while degrading lignin and leaving the cellulose untouched [14] and reports on fungi with unique proteomes and lignocellulose degrading activities [150] are evidence that these are challenges that can be overcome.

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