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Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels

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A biochemical platform holds the most promising route toward lignocellulosic biofuels, in which polysaccharides are hydrolyzed by cellulase enzymes into simple sugars and fermented to ethanol by microbes. However, these polysaccharides are cross-linked in the plant cell walls with the hydrophobic network of lignin that physically impedes enzymatic deconstruction. A thermochemical pretreatment process is often required to remove or delocalize lignin, which may also generate inhibitors that hamper enzymatic hydrolysis and fermentation. Here we review recent advances in understanding lignin structure in the plant cell walls and the negative roles of lignin in the processes of converting biomass to biofuels. Perspectives and future directions to improve the biomass conversion process are also discussed.

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Introduction

Higher plants produce the most abundant terrestrial biomass on earth. Plant cell walls, or lignocellulose biomass, are now considered to be a renewable resource for the production of biofuels through chemical or biological processing [1,2]. Scientists and engineers have long been engaged in developing an industrial-scale process that meets the challenges of producing biofuels in a manner that is cost effective and environmentally benign. A 'zero waste' process, meaning that all sugars or carbons fixed in biomass must be utilized efficiently [3], is the ultimate goal. Among current state-of-the-art technologies developed over past decades, a biochemical platform holds the promise of achieving these goals. In this process, polysaccharides in the plant cell walls are depolymerized to simple sugars that can then be fermented to ethanol or other biofuels by microbes.

In biomass, polysaccharides are 'protected' in the cell walls by lignin. Natural biomass decay is carried out by microbial communities in which polysaccharides are consumed by microbes as a food source, lignin is transferred from plants to soil as carbon storage [4]. As the structure of lignin is complex and variable, only a few microorganisms, including bacteria such as *Streptomyces* sp., and *Nocardia* sp. and basidiomycetes (brown-rot and white-rot fungi, respectively), are able to degrade lignin relying on the oxidative action of unspecific and extracellular enzymes, such as lignin peroxidase, manganese peroxidase, and laccase [4]. However, hitherto no evidence has shown any organism can use lignin as food source for life.

The fundamental principles of biomass deconstruction and conversion for biofuels production are the same as we have learned from natural microbial decay systems that are effective but slow; the biorefinery, however, must be designed to process biomass efficiently at a large scale. A thermochemical pretreatment step is therefore required to remove or delocalize lignin before the enzymatic hydrolysis of biomass. The overall efficiency of biomass conversion thus largely relies on improvement of pretreatment technologies, specifically the effectiveness of lignin modification [5••].

This review paper discusses recent advances in the understanding of molecular mechanisms of biological conversion of biomass to biofuels. Special focus is on the lignin structure, content, and distribution in the plant cell walls and their roles in affecting the efficiency of the biomass conversion processes, including chemical pretreatment, enzyme hydrolysis, and microbial fermentation.

Lignin in the plant cell walls

During plant cell growth, the primary wall (PW) is formed first; while differentiating, most cells are expanded and/or elongated, and the secondary wall (SW) layers are formed by depositing wall substances onto the inside of the PW. In mature cells, there are two types of SWs: the parenchyma-type SWs (pSW) are thickened walls in parenchyma, and collenchyma, which are normally found in living cells. The sclerenchyma-type SWs (sSW) are secondarily thickened walls in highly differentiated cells, such as tracheary elements and fibers, which are elongated and dead cells [5••]. The chemical composition of these walls varies dramatically in different cell types, in different tissues, and in different plant species. Some cells possess only non-lignified PWs. Cells that have thickened SWs

are usually lignified, consisting of a multilayered structure: from outside to inside are highly lignified compound middle lamellae (CML) containing middle lamellae and PW; a thin S1 layer; a thick, less-lignified middle S2 layer; a thin inner S3 layer; and a warty layer formed by lignin precursors [6]. Dry plants in general comprise 40–50% cellulose, 15–25% hemicelluloses, 5–10% other components (ash, minerals, etc.), and 20–25% lignin [7]. These lignified SWs account for the majority of plant biomass.

Lignin is the second most abundant biopolymer besides cellulose, consisting primarily of three units: guaiacyl (G), sinapyl (S), and p-hydroxyphenyl (H) units linked by aryl ether or C–C bonds. Traditionally, lignin is detected by histochemical staining or using ultraviolet light to excite the lignin aromatic structure to generate blue fluorescence. Vibrational spectroscopy is also widely used to characterize biomass chemistry; in particular, recently developed coherent Raman microscopy (CRM), which uses coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS), has provided semi-quantitative mapping of lignin and carbohydrates *in situ* based on their unique vibrational mode at 1600 cm^{-1} (the aromatic ring stretch) and 2900 cm^{-1} (C–H stretch), respectively (Figure 1). The coherent Raman signal generated by these non-linear processes is so high that a 2048 × 2048 pixel resolution image (at spatial resolution <300 nm) can be obtained within a few minutes [8,9].

The difference in lignin content in the wall layers is the result of the unique pathways of lignin synthesis during plant development. Lignin is synthesized through oxidative coupling of 4-hydrophenylpropanoids. This radical polymerization process can be either developmentally programmed or initiated by environmental factors, such as stress conditions. Lignification constitutes the last stage of cell division, expansion, and elongation before cell death. Lignin monomers are biosynthesized inside the cell membrane and then translocated to the cell wall via mechanisms that are still not fully understood. Nevertheless, lignification starts from the cell corner, accumulates in the CML, and gradually extends into the PW and the SW layers (i.e. S1, S2, and S3), resulting in the same trend of lignin content from high to low in these layers. The cell corner, being the junction of the CMLs, always has the highest lignin content. The adjacent lignified PW and S1 layer also have relatively high lignin concentrations. The S2 and S3 layers are further away from the lignification initialization sites and have less lignin content. The warty layer next to S3 is composed of highly cross-linked lignin precursors that are formed while the cell is in the final stage of lignification and death. The two types of SWs undergo different lignification processes. Taking maize as an example, while there is steady increase in biomass during the plant's vegetative growth phase, the lignin content stays at very low level. Only sSWs are lignified. A dramatic jump in lignin content is observed during the transition from the vegetative to the reproductive growth phase, which is mainly attributed to lignification of pSWs. During the reproductive growth phase, lignin steadily increases until plant senescence while the biomass experiences a slight drop and then remains constant (Figure 2). At the cell wall level, significant changes in lignin content during plant growth happen to pSW, which has almost no lignin during the vegetative growth phase but has high lignin content during the reproductive growth phase. In the mature plant, the sSWs are always fully lignified on both sides of the CML and warty layers, the pSWs are partially lignified, and there is a lack of the S3 layer and the warty layer.

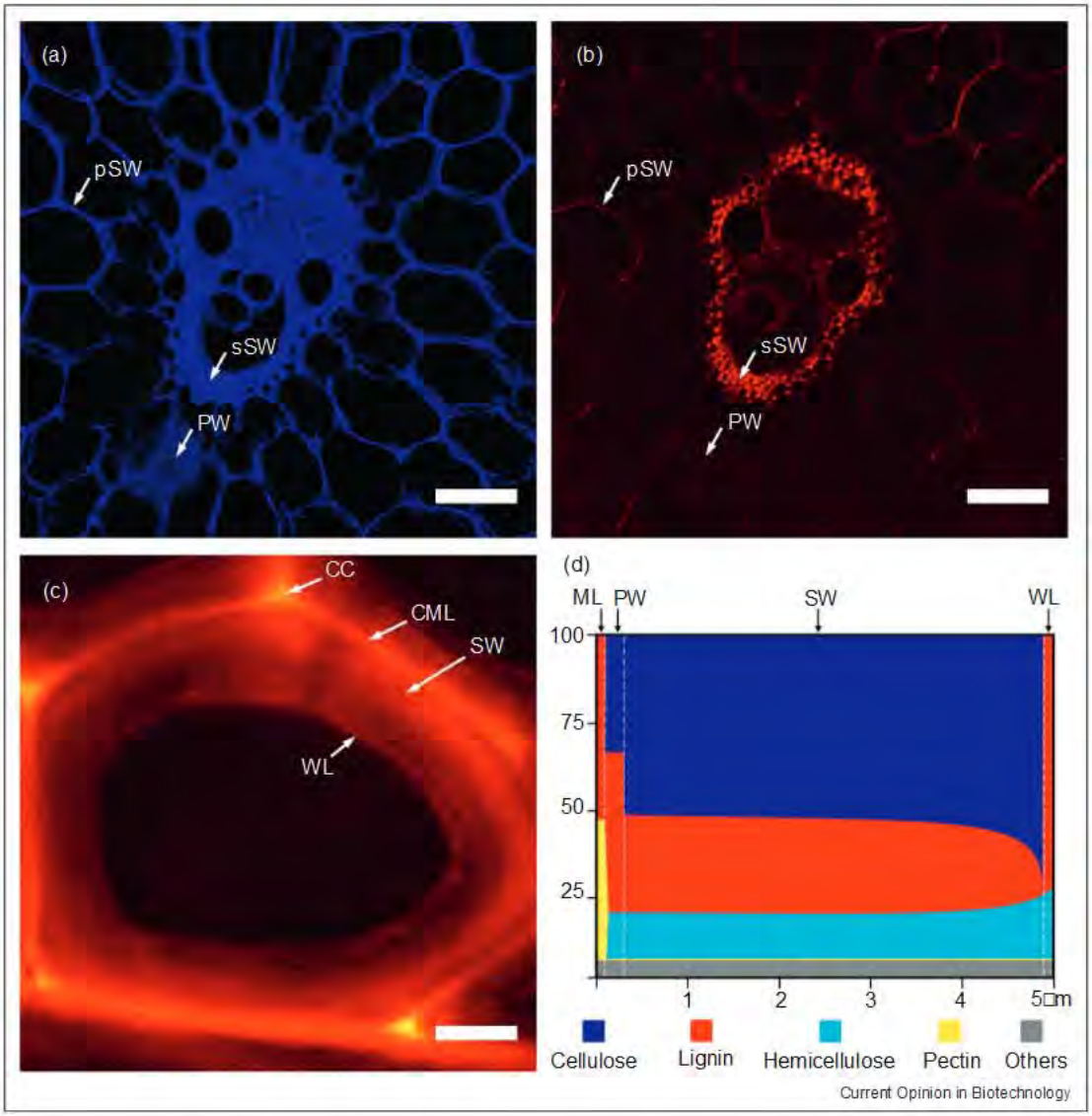
Lignin and pretreatment

The purpose of pretreatment is to make biomass amenable to enzyme hydrolysis of polysaccharides to fermentable sugars. Many pretreatment approaches have been developed empirically with a combined effort of reducing particle size by physical milling or steam expansion, elevating temperature, and applying acid, alkaline, or organic solvents to change the physical and chemical properties of biomass and improve enzyme digestibility [11]. However, some pretreatment processes may also result in reduction of the overall yield of fermentable sugars and generate compounds that hamper enzymes hydrolysis and fermentation microbes.

There is limited investigation focused on lignin properties that affect biomass susceptibility to pretreatment. It is well known that a given pretreatment approach can be more effective for one type of feedstock than for others. Studies on grass biomass that has different lignin phenotypes have demonstrated that lignin content, but not S/G ratios, is negatively correlated with the efficiency of delignification by alkaline hydrogen peroxide, indicating that the lignin's chemical composition may not be the main factor that affects pretreatment [12]. Although modification of lignin may not be the initially designated goal in some pretreatment processes, recent study has indicated that the key factor of pretreatment is indeed to maximize lignin removal and minimize polysaccharide modification, retaining the native-like structure of the plant cell walls to allow easy access by enzymes to polysaccharides and enable their rapid digestion [5••].

To access the polysaccharides in the lignified walls and to accomplish complete digestion by enzymes, the condensed lignin layers must first be broken down to allow pretreatment chemicals to penetrate and remove the mesh lignin networks in the SW. The condensed lignin layers are usually more resistant to pretreatment chemistry and require combined mechanical, temperature, and chemistry efforts, namely high severity. In plants, the inner face of the pSWs is 'opened' (non-lignified) whereas

Figure 1

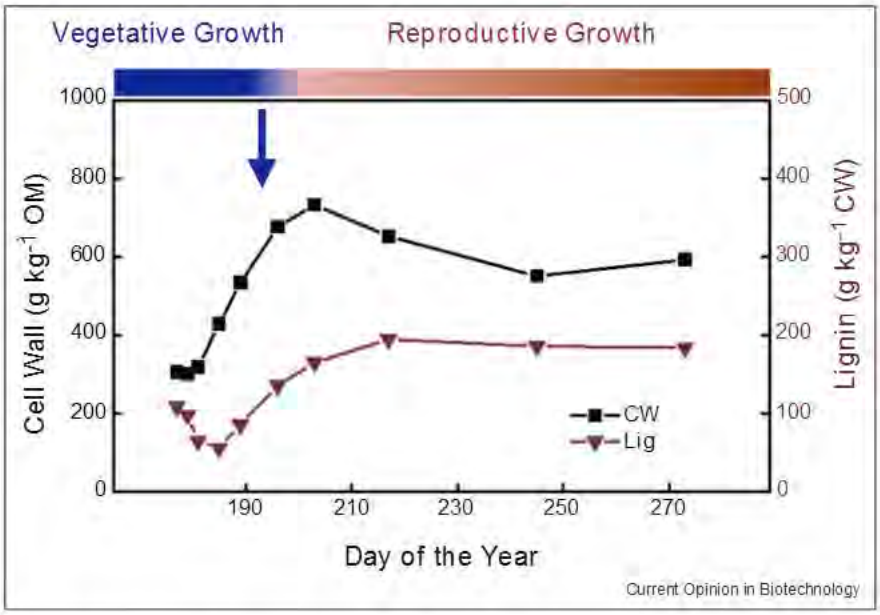


Cell wall types and chemical composition. PW: primary wall; pSW: parenchyma-type secondary wall; sSW: sclerenchyma-type secondary wall; CC: cell corner; CML: compound middle lamella; ML: middle lamella; WL: warty layer. Scale bars = 50 mm **(a,b)**, and 5 mm **(c)**. SRS micrographs of the vascular bundle area in maize stem cross section showing carbohydrates at 2900 cm^{-1} (a) and lignin at 1600 cm^{-1} (b). PWs are non-lignified, pSWs are partially lignified, and sSWs are fully lignified. (c) SRS micrograph of poplar tracheid wall cross section showing lignin content in wall layers. **(d)** Relative amount of major components across cell wall layers in the sSW. The high lignin concentration peaks in CML (middle lamella and PW) and the warty layer form a sandwich structure that encloses cellulose/hemicelluloses (mostly in the SW layer as shown in the figure) in between. 'Condensed lignin' refers to lignin in the CML and the warty layer where there are few or no polysaccharides; 'mesh lignin' refers to lignin in the SWs that forms molecular networks between the cellulose/hemicellulose lamellae.

in the sSWs, the SW is sealed between CML and the warty layer. Different strategies may be needed to treat different biomass feedstock (Table 1). For example, dilute acid is sufficient in grass biomass because there are large amounts of pSWs to allow acid penetration from the inner sides of the walls. The same method may not work in woody biomass because wood chips are composed predominately of sSWs, which require much higher severity (at 190 8C or higher for 30 min or longer retention time) or different pretreatment methods that combine physical (milling or steam explosion) and chemical (delignification) processes [13].

As shown in Figure 3, lignin is believed to form hydrophobic networks that are covalently linked to hemicelluloses by benzylether, benzylester, phenylglyside, and acetal type bonds. The lignin–hemicellulose and cellulose–hemicellulose layers are alternately located in the wall, forming

Figure 2



Maize cell wall (CW) biomass and Klason lignin (Lig) content during the vegetative and reproductive growth phases. The blue arrow indicates the approximate date of growth phase transition.
The data were reproduced from Ref. [10].

sandwich-like multi-lamellae structures. Theoretically, chemical cleavage of aromatic rings of lignin monomers, linkages between lignin units, and ester or ether bonds between lignin and hemicellulose could all release lignin from the polysaccharide network. Pretreatment, such as dilute acid at high temperature (160 8C and above), can hydrolyze glycosyl bonds in hemicelluloses so that lignin-carbohydrate complexes (LCCs) are formed and redeposit on the biomass surface as droplets, thus exposing cellulose. Other pretreatment methods directly remove lignin (Figure 3).

Lignin and enzyme hydrolysis

In untreated biomass, the non-lignified PWs are rapidly digested without pretreatment, but their contents in bulk biomass are negligible. The non-lignified pSWs collected before reproductive growth in maize were also found to be completely degradable whereas degradation is not observed in the fully lignified sSWs [14]. Lignin could be selectively bleached by dilute acid chlorite at room temperature, and cellulose and hemicelluloses would be retained nearly unchanged. All SWs were found to be digestible at a similarly rate as PWs [5••]. All these studies appear to suggest that the lignin content is the key factor negatively correlated with enzyme digestibility.

Table 1

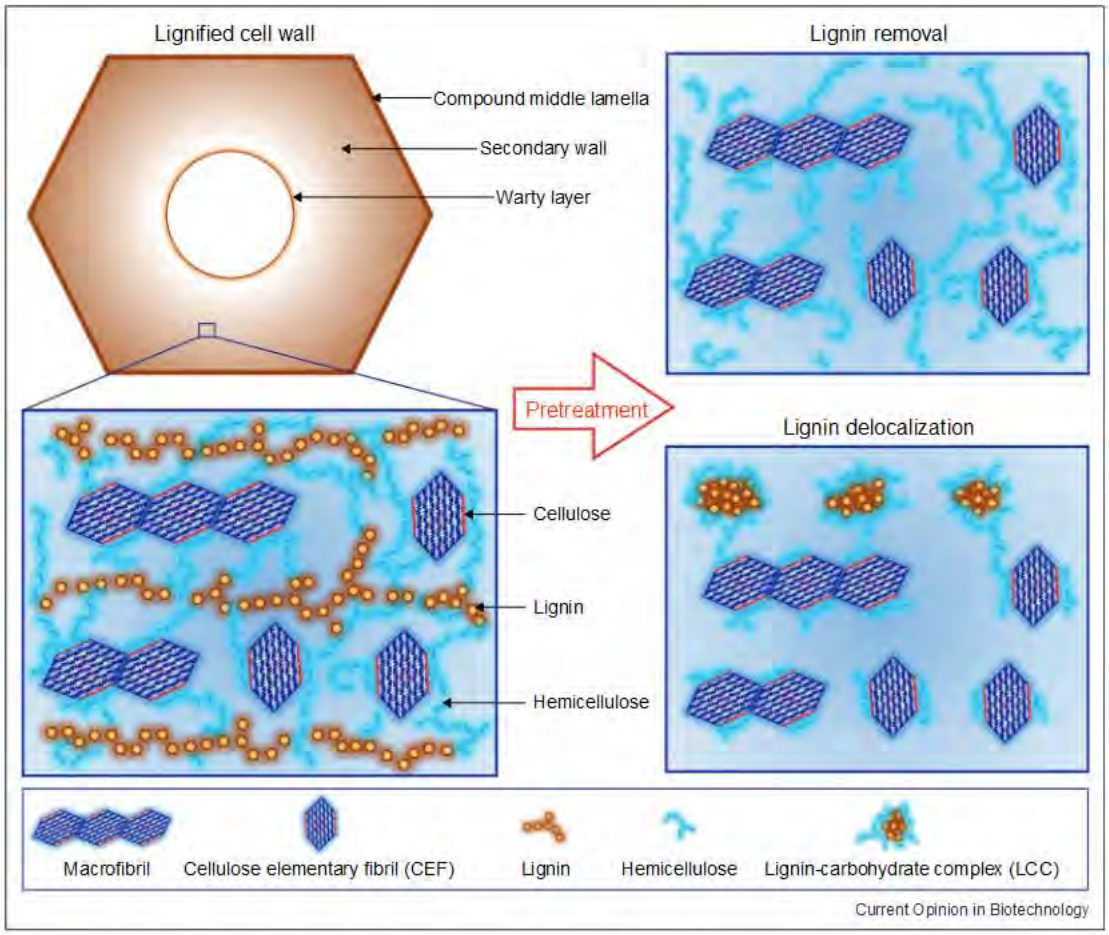
Wall types and rough total biomass and lignin content in grass and wood biomass

Wall types	Total biomass (lignin content)	
	Grass	Wood
PW	+ (no)[a]	Very low (no)
pSW	++ (no) Vegetative ++ (++) Reproductive	+ (no)
sSW	+++	+++++ (+++++)

[a] '+' indicates the relative content; 'no' indicates non-lignification.

Studies using enzyme labeling and high-resolution microscopy have demonstrated that in untreated cell walls, cellulases bind only to non-lignified walls, indicating that non-specific binding of the enzyme to native lignin is negligible. In the case of pretreated biomass, residual lignin normally forms LCC droplets or particles. For instance, in pretreatment in aqueous conditions, such as dilute acid, the LCCs may form micelle-like structures, in which lignin is the hydrophobic core and polysaccharides are displayed on the surface that could be attractive to non-productive binding of enzymes [15]. Enzyme binding to the LCCs therefore relies on the relative content of polysaccharide and its morphological structure. Depending on the pretreatment chemistry, lignin may or may not be chemically modified, and the composition of the resulting LCCs may contain nearly pure lignin or significant amounts of polysaccharides, mainly hemicelluloses. It has also been reported that lignin isolated from wood is more inhibiting to enzymes than that from herbaceous plants [16], and lignin isolated from pretreated biomass, such as by steam explosion, exhibited more inhibiting effects on enzymes than lignin isolated from non-pretreated raw biomass [17•].

Figure 3



Schematic structure of a lignified cell wall and possible changes by pretreatment. The sSW is enclosed by two condensed lignin layers (i.e. warty layer and CML), whereas the pSW lacks the warty layer. The prerequisite of enzymatic saccharification is the accessibility of enzymes to the polysaccharides that is physically impeded by lignin networks in the SW. Therefore, effective enzyme–cellulose interaction requires either effective removal, such as by lignin bleaching, or delocalization of lignin, such as melting lignin into small LCCs from the active sites on cellulose microfibrils.

Studies on the influence of lignin composition, that is, S/G ratios [18••], have shown statistically complicated, sometimes controversial results.

Complete removal of lignin from biomass results in extremely digestible material [5••]. However, lignin removal must be performed at low temperature to avoid sugar degradation and to maintain the integrity of the cell wall architecture. Delignification of pretreated biomass with removal of most hemicellulose may result in significant reduction of enzyme digestibility [19], which could be attributed to the collapse and aggregation of the cellulose microfibril network, both of which reduce efficient enzyme penetration and rapid digestion.

Lignin and fermentation

Potential pretreatment byproducts, including organic acids, furan derivatives, and phenolic compounds generated from pretreatment methods, such as dilute acid and ammonia fiber expansion, were reported to inhibit downstream microbial fermentation, causing membrane disruption, DNA mutation, intracellular pH imbalance, enzyme inhibition, among others [20–22]. The influence of these byproducts on fermentation organisms has been primarily carried out by next-generation sequencing technology and systems biology, focusing on understanding the toxicity mechanisms in model species, such as yeast *Saccharomyces cerevisiae* and bacteria of *Escherichia coli* and *Zymomonas mobilis*, for model inhibitors such as acetate and furfural [22–24,25••,26•,27••,28–30]. However, investigation of lignin byproducts has still been neglected because of its complicated chemistry and trace amount in hydrolysates, which are difficult to detect and study except for the known lignin monomers [22,23,31,32].

Lignin degradation byproducts may not be the major concern in the fermentation process due to their small amounts in the hydrolysates generated by current

pretreatment processes, such as dilute acid, and are often overlooked due to larger amounts of common inhibitory compounds, such as acetate and furfural. The potential synergistic effect of these chemicals and their functions on gene regulation may, however, affect the performance of fermentation microbes in a way we do not understand yet. For example, plant phenolic acids can inhibit phytopathogens, and in return phytopathogens control their pathogenic gene expression tightly in response to plant host phenolic compounds [33,34].

Individual tolerance genes have been identified as a byproduct of specific pretreatment methods. However, combining these tolerance-improving genes with individual inhibitors unnecessarily results in further enhancement of the capability of microbial tolerance [35]. Furthermore, due to the relatively labor-intensive nature of these investigations, and their low efficiency, they are currently limited to using certain model strains, such as *E. coli* and *S. cerevisiae* [36,37]. To fully understand the impact of pretreatment and saccharification processing on microbial performance, the full spectrum of chemicals present in hydrolysates, especially the lignin byproducts that may affect microbial biocatalyst gene expression, should receive more attention through systems biology and traditional physiology studies [25••,38]. Nevertheless, with the explosive accumulation of systems biology data and the rapid evolution of metabolic engineering and synthetic biology technologies, we can foresee that in the future we can construct whole series of microbial biocatalysts with excellent industrial functionalities (e.g. high yield, productivity, robustness) easily through rational design specialized for different biofuel and biochemical production processes using various lignocellulosic feedstocks, although challenges need to be overcome [39].

Perspectives and future directions

A short-term goal of lignocellulosic biofuel research and development would be a process that is comparable with corn ethanol production. To meet the technical challenge, lignocellulose must be pretreated to be digestible like starch. Recently, we have demonstrated that removal of lignin under mild conditions (i.e. diluted acid chlorite at room temperature) allows complete enzyme digestion within several hours using a commercial cellulase mixture [5••], which is comparable to starch saccharification. We therefore suggest that pretreatment should be developed to maximize lignin removal while maintaining cellulose and hemicellulose intact. However, pretreatment methods aiming at lignin removal are usually expensive and sometimes raise environmental concerns. Alternatively, genetically modified energy plants that have reduced lignin content and can grow in marginal land would also meet these challenges.

Although it has been widely accepted that reducing the lignin content in plant cell walls by genetically downregulating lignin will improve digestibility, it is not practical to dramatically reduce lignin because plants need a minimum amount of lignin to maintain their normal biophysical functions. Other biochemical factors such as the chemical structure of lignin or the S/G ratio have been explored in alfalfa, *Arabidopsis*, maize [40••], and tobacco. The genetic mutations induce complex responses in plants, and their benefit to the purpose of biofuel yield is still under debate. Some results suggest lower S/G ratios at the same lignin content would be beneficial for cell wall degradability while others suggest the opposite. But no matter how much the S/G ratio fine tunes digestibility, those results unanimously support that low total lignin content in plants always favors higher cell wall degradability.

Lignin synthesis consumes precious energy that could be otherwise used to produce biomass. Lignin contains more energy than cellulose, and indeed it takes three times the energy equivalent of glucose to produce lignin [41]. Evidence from *Eucalyptus* and *Populus* has shown a clear negative correlation between plant growth and lignin content [42]. This is particularly useful for energy crops such as switch grass. Unlike wood, grasses contain similar amounts of pSW and sSW. The amount of lignin in grass increases dramatically when the plant transits to the reproductive growth phase, while at the same time the amount of carbohydrate decreases a little. For bioenergy purposes, to obtain more usable biomass with less lignin, it is particularly favorable to collect biomass right before the plant makes the transition from the vegetative growth phase to the reproductive growth phase. This is not as useful for wood, however, as wood usually contains mainly sSW, which are always lignified. Table 1 shows the relative amount of cell wall types in wood and grass and their lignin content. Because biomass cell walls for cellulosic biofuel are primarily from vegetative tissues, recent plant biological studies have discovered regulators that can increase or prolong vegetative meristem activities or delay the transition from the vegetative growth phase to reproductive growth phase [43]. Both of these genetic modifications will lead to higher vegetative tissue content.

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