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Recent advances in understanding the role of cellulose accessibility in enzymatic hydrolysis of lignocellulosic substrates

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Cellulose accessibility has been proposed as a key factor in the efficient bio-conversion of lignocellulosic biomass to fermentable sugars. Factors affecting cellulose accessibility can be divided into direct factors that refer to accessible surface area of cellulose, and indirect factors referring to chemical composition such as lignin/hemicellulose content, and biomass structure-relevant factors (i.e. particle size, porosity). An overview of the current pretreatment technologies special focus on the major mode of action to increase cellulose accessibility as well as multiple techniques that could be used to assess the cellulose accessibility are presented in this review. The appropriate determination of cellulose accessibility before and after pretreatment can assist to understand the effectiveness of a particular pretreatment in overcoming lignocellulosic recalcitrance to improve substrate enzymatic digestibility.

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Introduction

Research into the bioconversion of lignocellulosic biomass to fuels and chemicals has attracted much more interest over last few decades, due to the increasing global energy demand and growing concerns about energy security, rural development, and increasing costs as well as environmental impact associated with nonrenewal, non-degradable chemical production [1]. Lignocellulosic biomass, composed of cellulose, hemicellulose, and lignin as shown in Figure 1, is one of the few resources that can facilitate large-scale, sustainable production of the substantial volumes of biofuels and will play a key role in shifting world's dependency away from fossil fuels [2]. Currently the biological bioconversion of biomass to biofuels generally includes five main steps: biomass collection, pretreatment, enzymatic hydrolysis, fermentation, and distillation/rectification/dehydration to meet fuel specifications [3]. However, this bioconversion process is significantly hindered by innate biomass recalcitrance, which refers to the complex characteristics of lignocellulose to protect its carbohydrates from degradation by enzymes [4,5]. Although the molecular mechanisms of biomass recalcitrance are still not completely clear, the accessible surface area of exposed cellulose has been proposed as a particularly important factor [6,7•]. Pretreatment is known to render biomass more accessible to cellulase by altering the chemical compositions or physical structures of biomass [8].

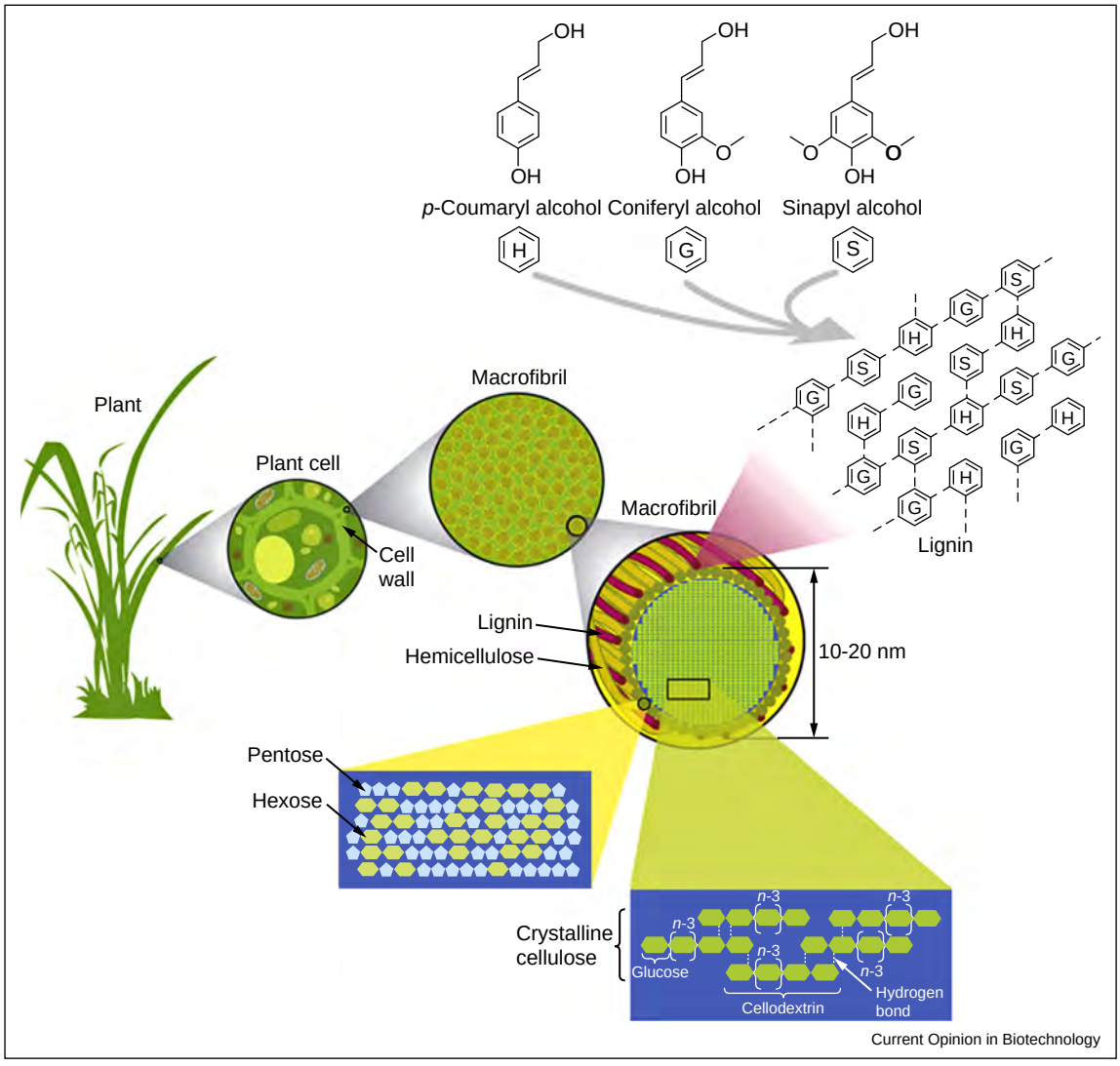
This review highlights recent advances in understanding the fundamentals of biomass recalcitrance with a special focus on the role of cellulose accessibility in enzymatic hydrolysis. Accordingly, the scope of this review covers factors affecting cellulose accessibility, methods being used to increase cellulose accessibility as well as the current studies utilizing multiple analytical techniques to characterize cellulose accessibility change before and after pretreatment by measuring relevant characteristics such as surface area, pore size/volume distribution.

Cellulose: structure, accessibility and enzymatic hydrolysis

The main component of lignocellulose is cellulose, a β(1-4)-linked chain of glucose molecules, which makes up 15–30% of the dry biomass of primary and up to 40% of the secondary cell wall. Cellulose unit, known as elementary fibril which is believed to contain ~36 β-D-glucan chains, coated with other non-cellulosic polysaccharides to form microfibrils, which are then cross-linked by hemicellulose/pectin matrixes to form macrofibrils that mediate structural stability in the plant cell wall [9].

The intimate contact between the cellulose and cellulase is the prerequisite step for enzymatic hydrolysis to occur, thus the surface area of cellulose is a critical factor for enzymatic hydrolysis yield and rate [10,11,12•]. Surface area of substrate can be divided into interior surface area which is essentially reflected by biomass porosity, and exterior surface area which is largely determined by particle size [4,13]. It has been show that cellulase accessibility to cellulose is mainly through the pores in the cell wall rather than substrate external surface, and more specifically, approximately over 90% of the substrate enzymatic digestibility is contributed by the accessible pore surfaces [12•,14,15]. There are several scales of

Figure 1



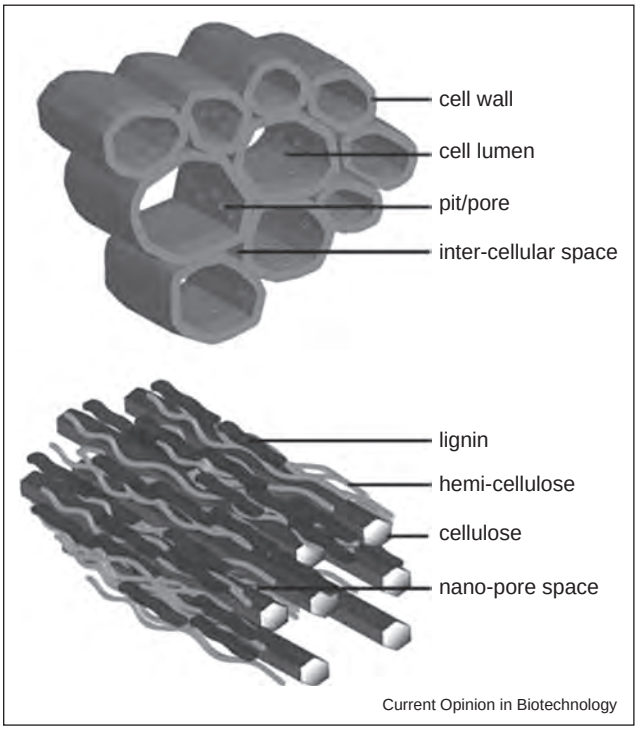
Structure of lignocellulose. Reproduced with permission from Ref. [3].

porosity in biomass from the cell lumen to the nano-pores between coated microfibrils as shown in Figure 2 [16]. The cell lumen, represents the largest scale of porosity, is not a critical barrier for enzymes because its size is normally in the range of tens of micrometers. Pits are regions in the cell wall where the secondary cell wall is absent and an open pore is maintained between adjacent cell lumen. They are only 20–100 nm and still do not represent the most fundamental barrier to enzymes. Clearly, a fundamental barrier to effective enzymatic hydrolysis is the accessibility of a reactive cellulose surface. Carpita *et al.* [17] estimated the architecture of plant cell wall pores to be approximately 5–10 nm in diameter which is too small to allow significant diffusion of enzymes. Transport phenomena suggest that pore size should be at least in the range of 50–100 nm to allow sufficient penetration of enzymes [16]. Many researches have indicated a positive relationship between interior surface area and enzymatic hydrolysis rate [14,15]. Earlier work by Grethlein [18] reported a linear correlation between the initial hydrolysis rate of steam pretreated hardwood and the pore volume of the substrate accessible to a nominal diameter of 5.1 nm representative of the diameter of cellulase.

Analytical techniques used to determine cellulose accessibility

One of the classic techniques to measure the specific surface area is the Brunauer–Emmett–Teller (BET) method using nitrogen adsorption [20]. However, it requires prior drying of the substrate which makes it typically less effective due to water removal from

Figure 2



Cartoon depiction of several scales of porosity from the cell lumen to the nanopores between coated microfibrils. Reproduced with permission from Ref. [19].

nonrigid porous materials could produce partial irreversible collapse of pores. Measurement of porosity has been frequently used as an alternative to represent the amount of accessible surface area of substrate, and pore size analysis are usually based on the assumption that biomass pores are cylindrical in shape [21]. Solute exclusion, a widely used method to investigate the pore characteristics of the lignocellulosic substrates, is based on the measured accessibility of pores to various sizes of non-interacting probe molecules such as dextran. Wang *et al.* [12•] evaluated the cellulose accessibility of a set of hornified pretreated lodgepole pine using solute exclusion, and reported that 24 hour air drying in a humidity controlled environment at 25°C can decrease the surface area that available to solute of 5.1 nm diameter from ~22 m^2/g to ~17 m^2/g. Another promising approach developed recently for quantitative determination of total substrate accessibility to cellulases relies on the adsorption of a non-hydrolytic fusion protein containing cellulose-binding module (CBM) and fluorescent protein (TGC) which have very similar molecular size to that of cellulase enzymes [12•]. However, these proteins also bind unspecifically to lignin and therefore require a step using BSA to block the lignin prior the adsorption of cellulase enzymes. An alternative approach to examining pore size employs direct dyes such as Simons' stain as a potentially useful semi-quantitative method for estimating the total available surface area of lignocellulosic substrates [10,22,23,24•]. In addition, techniques involved using nuclear magnetic resonance (NMR) are also valuable diagnostic tools in terms of porosity measurement, including NMR cryoporometry and relaxometry [24•,25–27]. A summary of these analytical methods for characterization of cellulose accessibility for lignocellulose substrates is presented in Table 1.

It is not straightforward to measure the porosity in biomass because properties such as dimension, geometry, and connectivity should be all considered. For example, the 'ink-bottle' effect which refers to a large pore connected to a small opening, can limit the accessible surface area of the substrate, therefore should be considered during a pore size measurement, and most of the techniques applied on biomass such as solute exclusion, Simons' stain all failed to account this effect. In addition, different techniques can give considerably different results, due to the differences in the principles of measurement between the techniques. The mean pore diameter of pine kraft fibers was determined to be around 3 nm using solute exclusion, while it was significantly higher when measured by a NMR technique, about 13 nm [28].

The role of hemicellulose and lignin in cellulose accessibility

It has been suggested that increasing cellulose accessibility depends on not only how much total biomass was removed but also what component with a specific structure and from where it was removed [33••]. Hemicellulose, which is generally found on the outer surface of cellulose fibers but is also diffused into the inter-fibrillar space through fiber pores, has been proposed to act as a physical barrier that limits the cellulose accessibility. Therefore, the addition of accessory enzymes such as xylanase during enzymatic hydrolysis can increase the cellulose accessibility as a result of xylan solubilization. A recent study by Hu *et al.* [34] also reported this significant improvement in cellulose accessibility indicated by Simons' stain is due to the increase in fiber swelling and fiber porosity caused by synergistic interaction of the xylanase and cellulase. Besides xylan removal, the effect of side-chain components such as acetyl groups on cellulose accessibility has been also investigated recently, indicating that acetyl groups may restrict cellulose accessibility by inhibiting productive binding through increasing the diameter of cellulose chain or changing its hydrophobicity [35,36].

Although removing lignin has been shown to increase yield of enzymatic hydrolysis in most current studies, the direct effect of lignin removal on cellulose accessibility is not fully clear because lignin reduces the effectiveness of

Table 1

Summary of analytical methods for characterization of cellulose accessibility for lignocellulose substrates

Techniques	Analytical background and procedure	Select advantages and disadvantages	References
Nitrogen adsorption	Nitrogen passes readily though cell walls and its uptake provides a good general measure of total surface area. Samples were dried, degassed, and then cooled in the presence of nitrogen gas, allowing nitrogen as to condense on the surfaces and within the pores. The quantity of gas that condensed was determined from the pressure decrease after the sample was exposed to gas, and the surface area was calculated using Brunauer–Emmett–Teller (BET) model that relates the gas pressure to the volume of gas adsorbed.	Advantages: Accurate, quick and robust method for determining the surface area accessible to nitrogen Disadvantages: Measurement requires a prior drying of the substrate which makes it typically less effective due to the partial irreversible collapse of pores Small size of nitrogen cause over-estimation of cellulose accessibility	[48,49]
Mercury porosimetry	Similar to nitrogen adsorption, dried and degassed samples were introduced into a chamber surrounded by mercury with pressure on the mercury gradually increased to force mercury into the pores. Relationship between pore diameter and applied pressure was given by the Washburn equation. The volume of mercury entering the pore was measured as the pressure increased, indicating the cumulative volume of all available pores of radius equal to, or greater than a corresponding pore diameter.	Advantages: Allows the pore size analysis to be undertaken over a wide range of mesopore–macropore widths Provides a wide range of information, e.g. pore size distribution, total pore volume, specific surface area, tortuosity, permeability, fractal dimension. No other porosity characterization technique can achieve this Disadvantages: Measurement requires a prior drying of the substrate Measures the largest entrance towards a pore, but not the actual inner size of a pore	[29,30]
Solute exclusion	Solute exclusion technique is based on the accessibility of probe molecules to the substrate pores of different sizes. A known concentration of a solute molecule solution is added into the swollen substrate. The probe molecule solution was then diluted by water contained in the initial substrate. The water presented in the pores that was not accessible to the probe molecules will not contribute to the dilution. As a result, the substrate pore size and volume distribution can be determined using the concentration of a set of different solute solutions with various molecule sizes.	Advantages: Measurement can be done in wet state quantitatively Disadvantages: Laborious, unspecific to cellulose, does not account for the external surface area Not an acceptable tool for determination of absolute pore size and volume distribution Effected by pore shape and osmotic pressure	[18,31]
Simons' stain	Simons' stain evaluates the large-to-small ratio of a substrate by applying two dyes with different color, molecular size and cellulose binding affinity. Samples were treated using a serious of mixed solution of orange and blue dye with increasing concentrations. The maximum amount of dye adsorbed to the lignocellulosic substrates was calculated using the Langmuir adsorption equations. The ratio of adsorbed orange and blue dye, a value used to estimate the relative porosity and assess the overall accessible surface area, can be then calculated.	Advantages: Measurement can be done in wet state Relatively fast, simple and sensitive Measure both interior and exterior surface area Disadvantages: Effected by pore shape and tortuosity Not fully quantitative	[13,32]
Protein adsorption	Quantitative determination of cellulose accessibility to cellulase based on the Langmuir adsorption of a fusion protein containing a cellulose-binding module and a green fluorescent protein. Protein adsorption on cellulose usually conducted in a typical enzymatic hydrolysis buffer solution, and the protein adsorption on the solid surface can be calculated by the Langmuir equation.	Advantages: Perfectly applied in enzymatic hydrolysis process due to the exist of a cellulose-binding module as cellulase has Probing molecule have a very similar molecular size to that of cellulase enzymes Disadvantages: Total exposed surface to the probe molecules include some non-cellulosic surface, for example, lignin.	[12•]

Table 1 (*Continued*)

Techniques	Analytical background and procedure	Select advantages and disadvantages	References
NMR cryporometry	Cryoporometry is a technique for determining pore size distribution that takes advantage of the fact that small crystals formed from liquid within pores melt at a lower temperature than bulk liquid known as melting point depression caused by enthalpic interaction with the pore surface. Hydrated samples were cooled to negative temperature to completely freeze all the adsorbed water, and the intensity of the NMR signal which represents the amount of unfrozen water at a specific temperature was measured by a Carr–Purcell–Meiboom–Gill sequence (CPMG) as temperature increases to generate the melting curves. The melting point depression of the liquid can be related to the pore size through the Gibbs–Thompson equation	Advantages: Non-destructively and quantitatively determination of pore size distribution Measurement can be done in wet state Disadvantages: Pore size determination range is limited by the temperature control Expensive, requires complicated setup and long experiment time	[24•,25]
NMR relaxometry	NMR relaxation experiment can provide information pertaining to the molecular mobility within a porous system. The spin–spin (T_2) relaxation curve can be obtained via a CPMG sequence to investigate the changes in the nature of biomass–water interactions and subsequent accessibility. Basically, as the T_2 relaxation time increases, the degrees of freedom of water in the pores also increases, causing a decrease in the proportion of amount of water located at pore surface versus the pore interior. Therefore, in systems of increasing average pore size, the pore surface area to volume ratio will decrease and is therefore detected by an increase in the T_2 relaxation time. It is also well known that liquid molecules near a solid surface will have different spin–lattice (T_1) relaxation profiles from that of the bulk liquid because of the interactions at the solid–liquid interface. As a result, the observed average T_1 time of the adsorbed water could also reflect the surface area to volume ratio of the pores	Advantages: Non-destructively measurement, not affected by pore inlet size or shape Disadvantages: Expensive, requires complicated experiment setup	[24•,27]

enzymatic hydrolysis by limiting the cellulose accessibility as well as by binding cellulase unproductively, and the relative contribution of these two roles of lignin is not yet fully understood. A recent study reported that the presence of lignin may not directly occlude cellulose present in lignocelluloses but rather impact cellulase action indirectly by its association with xylan [37]. Kumar and Wyman also reported that delignification of corn stover greatly enhanced enzyme effectiveness but had a very limited effect on cellulose accessibility, indicating that lignin did not directly control cellulose accessibility but restricted xylan accessibility which in turn controlled the access of cellulase to cellulose [35].

Increase of cellulose accessibility via non-hydrolytic proteins

In very recent years, several cellulolytic organisms have been shown to produce non-hydrolytic proteins that could be used as cellulase activity enhancement factors due to its ability to deagglomerate the cellulose manifested as dispersion of the microfibrils, loosening of the macrofibrils, swelling and roughening of lignocellulosic substrates, thereby increasing the cellulose accessibility. These non-hydrolytic disruptive proteins could be categorized into two distinct groups based on their catalytic mechanisms [10]. For example, proteins with uncharacterized catalytic function including Expansins, Swollenin, and Loosenin are thought to increase cellulose accessibility mainly through disruption of the hydrogen bonding network of the substrate. Recently, fungal-derived, copper-dependent polysaccharide monooxygenases (PMOs), formally known as GH61 proteins, have been shown to catalyze the oxidative cleavage of glycosidic bonds on the surface of cellulose without requiring separation of a glucan chain, increase the substrate accessibility for hydrolytic enzymes [38].

Increase of cellulose accessibility via different biomass pretreatments

To date, numerous physical or chemical pretreatment methods have been developed to overcome biomass recalcitrance, including dilute acid (DAP), hot water, lime, organic solvent, ionic liquid (IL) and ammonia fiber expansion (AFEX). The changes in lignocellulosic structure during these commonly applied pretreatment technologies have been recently reviewed by Hu and Ragauskas [39]. Although the mechanism of each pretreatment is different, the final objective is always the same — increasing cellulose accessibility. The major mode of action to increase the cellulose accessibility by different pretreatments is summarized in Table 2.

The increase of cellulose accessibility by hot water pretreatment, steam explosion and DAP is mainly due to the removal of hemicellulose [24•,41,42,49], while organosolv pretreatment increases cellulose accessibility mainly

Table 2

Summary of major mode of action for different pretreatments in terms of cellulose accessibility increase

Pretreatment	Major mode of action[a]	References
Mechanical	Reduction of particle size associated with increase of external surface area	[40]
Hot water	Preserving most of the cellulose Significant removal of hemicellulose Partially depolymerization of lignin Increase of plant cell wall pore size/volume	[7•,8,41]
DAP	Nearly complete removal of hemicellulose Significant disruption and redistribution of lignin Increase of plant cell wall pore size/volume	[7•,8,24•,27]
Steam explosion	Reduction of particle size associated with increase of specific surface area Significant removal of hemicellulose Partial transformation of lignin Significant expansion of pore size and increase of pore volume caused by explosive decompression	[8,24•,42]
Alkali	Significant removal of lignin Significant removal of acetyl groups and uronic acid substitutions on hemicellulose Swelling of cellulose leading to an increase of internal surface area	[8,39,43]
AFEX	Ammonolysis of lignin–carbohydrate complex ester linkages, solubilization and relocation of cell wall extractables leading to the formation of nanoporous, interconnected tunnel-like networks Rapid pressure release leading to the formation of large pores at the middle lamella cell wall	[8,44••]
Organosolv	Significant removal of lignin and hemicellulose Increase of accessible surface area and pore volume	[45,46]
Ionic liquid	Regeneration of nearly complete amorphous cellulose Disruption of inter- and intro-molecular hydrogen bonds resulting in the increase of accessible binding sites of cellulose for cellulase	[47,48]

[a] This section only focuses on the major mode of action of each pretreatment to increase cellulose accessibility.

by removal of lignin as well as hemicellulose [45,46]. Lignocellulosic structure of biomass simultaneously underwent fragmentation and swelling during DAP with fragmentation releasing small components, thereby enlarging the specific surface area. However, with the pretreatment time extended, the swelling behavior of biomass became more drastic, resulting in a much lower specific surface area. Chen *et al.* [20] reported that the specific surface area of DAP sugarcane bagasse decreased from 2.38 m^2/g to 0.98 m^2/g as the pretreatment time increased from 5 min to 10 min. A decrease in molecular weight of lignin during DAP, its hydrophobicity, and the surface tension effects of water can cause the deposition of spherical lignin droplets on the fiber surface, which increases the pore size for the enzymes to diffuse into and out of the cell-wall matrix, but at the same time it also significantly reduces the surface area upon which enzymes can productively bind [50,51]. However, this limitation in cellulose accessibility could be overcome by high enzyme loadings, delignification or treatments such as neutral sulfonation that increase lignin's hydrophilicity by incorporating sulfonic acid groups onto lignin [52]. It was also found that the near complete removal of xylan and lignin by DAP could result in decreased cellulose accessibility possibly due to the aggregation of adjacent cellulose microfibrils [53]. In contrast, AFEX pretreatment improved cellulose accessibility via cleaving lignin–carbohydrate ester linkages, partially solubilizing cell wall extractables and relocating these extractables to cell wall surfaces, thereby creating interconnected tunnel-like networks of nanoporous structures with sizes from 10 to 1000 nm, as visualized by TEM and 3D-electron tomography [44••]. Alkaline pretreatment increases the cellulose accessibility via removing lignin as well as some acetyl groups and various uronic acid substitutions on hemicellulose that lower the accessibility of enzyme to the cellulose [54]. IL effectively dissolves the highly ordered hydrogen bond in cellulose fibers causing the increase in accessibility much more effective than traditional pretreatments [55,56]. Li *et al.* [48] reported a significant increase in the BET surface area from 0.7 to 15.1 m^2/g, which is 21.6 times greater after IL pretreatment using 1-ethyl-3-methylimidazolium acetate for corn stover at room temperature.

One of the most critical challenges that must be addressed in order for lignocellulosic biofuels to become commercially available is to develop cost-effective pretreatments. Steam explosion and hot water pretreatment makes use of water and therefore has the lowest recycling and environment cost. The reactor system for DAP is more costly than hot water pretreatment reactor, and the acid neutralization and recovery after pretreatment also increases the costs. As an alkaline pretreatment, lime

pretreatment can be performed at low temperature which significantly reduces huge energy and cost demand required to maintain high thermal steady conditions as well as the use of pressured vessels [57]. The relative high cost of organic solvents used in organosolv pretreatment makes it much more expensive than other leading pretreatment processes, however, it also potentially lower enzyme costs by separating lignin before the enzymatic hydrolysis. As a very effective pretreatment in terms of cellulose accessibility, ionic liquid pretreatments currently suffer significant challenges that stand in the way including the high cost associated with the use of ionic liquid as well the subsequent requirement of ionic liquid recovery and recycling [58].

In conclusion, all pretreatments significantly increase the accessible surface area of cellulose via various mechanisms such as lignin and hemicellulose removal/redistribute, particle size reduction, and pore expansion. Delignification through alkaline pretreatment has been shown less effective than hydrolysis of hemicellulose using acid in terms of cellulose accessibility increase [33••]. In fact, DAP is probably one of the most effective pretreatment techniques among the traditional pretreatments due to its ability to redistribute the lignin and significant pore expansion besides nearly complete removal of hemicellulose. The relatively new IL pretreatment is probably the most effective pretreatment techniques to increase cellulose accessibility, though facing significant challenges, has receiving growing interest from the biofuels community. In our opinion, the ideal pretreatments should economically minimize the recalcitrance and at the same time maintaining the integrity of fermentable sugars.

Genetic modification of biomass feedstock with low recalcitrance

Recently, genetic manipulation of biomass feedstock has been mainly focused on changing the cell wall components and structures to improve cellulose accessibility. One of the strategies is to develop low-lignin transgenic plants with altered lignin structures. Research on the molecular mechanisms regulating lignin biosynthesis in biomass feedstock, such as switchgrass has just started in recent years. Transgenic switchgrass with a down-regulated caffeic acid O-methyltransferase (COMT) gene in the lignin pathway revealed a normal growth phenotype, reduced lignin content, showed significantly improved saccharification efficiency by 29–38% without pretreatment [59]. Another lignin biosynthesis gene for switchgrass, *cinnamyl-alcohol dehydrogenase* (CAD) was also recently founded, and the down-regulation resulted in a decreased lignin content of switchgrass that potentially enhances the biofuel production [60]. Furthermore, a very recent study demonstrated that overexpression of PvMYB4 gene, a general transcriptional repressor of the phenylpropanoid/lignin biosynthesis pathway, could lead to high yield ethanol production [61•]. Altering hemicellulose levels and their side chain is another main approach to genetically modify the plants to increasing cellulose accessibility. Silencing of the *PoGT47C* gene in poplar, a glycosyltransferase homologous to *Arabidopsis FRA8* involved in hemicellulose biosynthesis, has been reported to reduce the xylan content and increase the glucose yield [62].

Perspectives and future directions

Undoubtedly, biofuels derived from biomass will play a key role in reducing the world dependence on fossil fuels. In the US, lignocellulosic biomass, in the form of forest, agricultural residues, and bioenergy crops, have the potential to provide around 500 million dry tons of biomass at $60/ton or less in 2012, and thus replace around 15% of current petroleum based transportation fuels [63]. Therefore, better understanding of the mechanisms contributing to biomass recalcitrance is critical but at the same time also very difficult due to the fact that lignocellulosic biomass is a multi-scale, complex and highly heterogeneous material. Research studies conducted in an effort to understand and overcome biomass recalcitrance frequently fail to take into account the integrated effect of an array of cell wall characteristics, thus the data gathered may be limited in application.

Several recent studies dealing with accessibility measurement of lignocelluloses often use only one analytical technique whereas a broader suite of techniques may provide a more definitive analysis. For example, nitrogen adsorption alone only gives the total specific surface area, and the Simons' stain determines the total accessible lignocellulosic surface area. Protein adsorption using a cellulose binding module could determine the cellulose accessibility to cellulase which represents the accessible cellulose surface area. Thus, a combination of these three techniques can provide a better picture of the surface properties of lignocellulosic substrates including information about total specific surface area, total accessible lignocellulosic surface area, and total accessible cellulosic surface area.

In conclusion, costs associated with enzymes and pretreatment are the major barriers that hind the broad industrial conversion of cellulosic biomass to biofuels. The costs associated with enzyme loadings could be minimized by developing novel cost-effective pretreatments that maximize the cellulose accessibility, while the need for expensive and harsh pretreatments can be reduced by developing genetically modified low recalcitrant energy plants. All these challenges are difficult to overcome by any individual investigator and will require broad multi-disciplinary approach in genetics, process chemistry, biotechnology and engineering.

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