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Enhancing Cellulose Accessibility to Cellulase by Ionic Liquid Pretreatment: Interplay Between Cellulose Accessibility and Glucan Digestibility

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pathogens, and harsh environments. This resilient lignocellulose structure, in turn, thwarts bioprocessing by preventing cellulolytic enzymes access to the cellulosic substrates and the subsequent release of sugars. Thus, bioprocessing requires a pretreatment process to disrupt the intimately bound polymers in the lignocellulose structure. However, pretreatment is one of the more costly steps in the bioconversion of lignocellulose. Typically, harsh pretreatment conditions are used that degrade sugars, generate inhibitors of enzymes and microbes, and condense lignin by-products. Ionic liquids, one type of cellulose solvent, partially dissolves lignocellulose and enhances cellulose accessibility to enzymes; these solvents enable efficient release of glucose with moderate reaction conditions (e.g., <160 C). In this chapter, we describe the effect pretreatment by ionic liquids (and other cellulose solvents) has on cellulose accessibility to cellulases (CAC). Understanding CAC enables us to measure the lignocellulose recalcitrance and optimize the pretreatment conditions to maximize sugar yield and other bioproducts for profitable biorefineries.

Introduction

Lignocellulose is a potential sustainable feedstock for the production of renewable biofuels and bioproducts. Nature embeds cellulose microfibrils within hemicelluloses and lignin, creating a robust nano-scale structure that is resistant to insects,

Lignocellulose as New Petroleum Supplement

The use of lignocellulose as a feedstock for fuels and chemicals provides a sustainable strategy to feed and fuel our society [1, 2]. Lignocellulose

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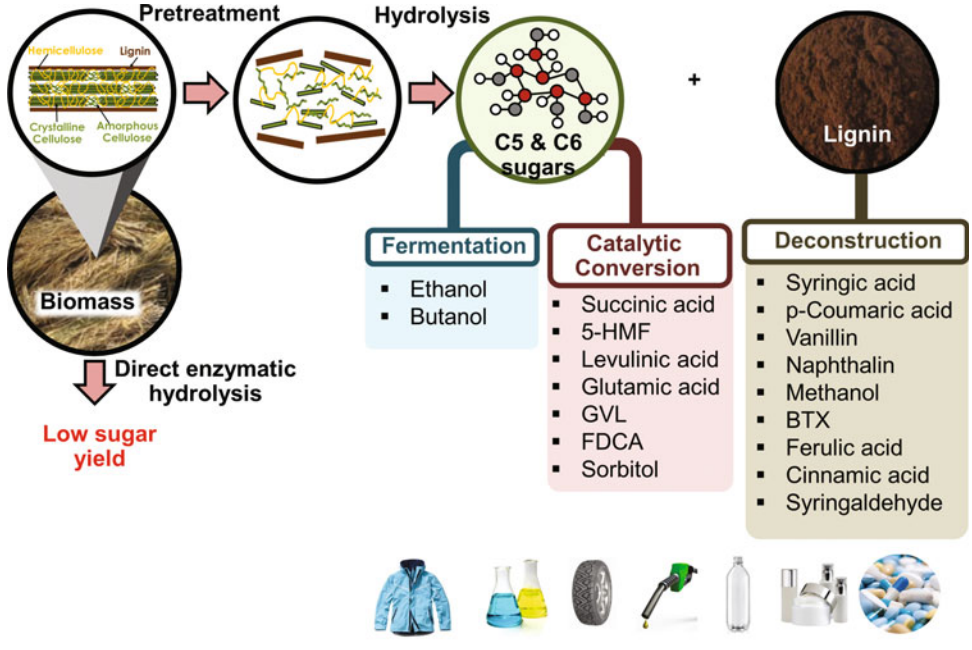
consists of three principal biopolymers: cellulose, hemicelluloses, and lignin. Cellulose, the most abundant polysaccharide, makes up ~30–50% of lignocellulose, whereas hemicellulose and lignin make up ~15–35% and 10–25% of lignocellulose, respectively [3]. Cellulose and hemicelluloses are good sources of C6 and C5 sugars, which are useful for the production of biofuels and bioproducts (Fig. 1). Lignin has good potential as a substitute for petroleum-based compounds in adhesives and as a renewable source of aromatic precursors, such as syringic acid, *p*-coumaric acid, vanillin, benzene-toluene-xylene, and ferulic acid. To feed and fuel our growing society, we cannot afford to let any part of the lignocellulose go unused. Hence, it is crucial to develop integrated processes that use both carbohydrates and lignin.

The biochemical pathway is commonly used in upgrading lignocellulose in the current biorefinery scheme. The biochemical pathway typically uses a combination of the pretreatment processes, followed by biological conversion. Thermochemical pathways such as pyrolysis and gasification typically require high temperature, pressure, and/or catalysts to produce products, such as 5-hydroxymethylfurfural (HMF), levulinic acid, succinic acid, γ-valerolactone (GVL), and 2,5-Furandicarboxylic acid.

Pretreatment Is a Key Step for Efficient Enzymatic Saccharification of Lignocellulose

The complex structure of lignocellulose makes it resistant to enzymatic conversion [4]. As a result, a pretreatment step is required for two major reasons (Fig. 2). First, cellulase enzymes bind non-specifically to the cellulosic (crystalline and amorphous celluloses) and noncellulosic fractions (hemicellulose and lignin) of lignocellulose. Cellulase bound to crystalline cellulose has a much lower hydrolysis rate and produces a lower yield compared to amorphous cellulose [5–8]. Moreover, the presence of hemicellulose and/or lignin



Enhancing Cellulose Accessibility to Cellulase by Ionic Liquid Pretreatment: Interplay Between Cellulose Accessibility and Glucan Digestibility, Fig. 1 Schematic of production of various bioproducts from lignocellulosic biomass. Note: 5-hydroxymethyl furfural (5-HMF), γ-valerolactone (GVL), 2,5-furandicarboxylic acid (FDCA), benzene-toluene-xylene (BTX)

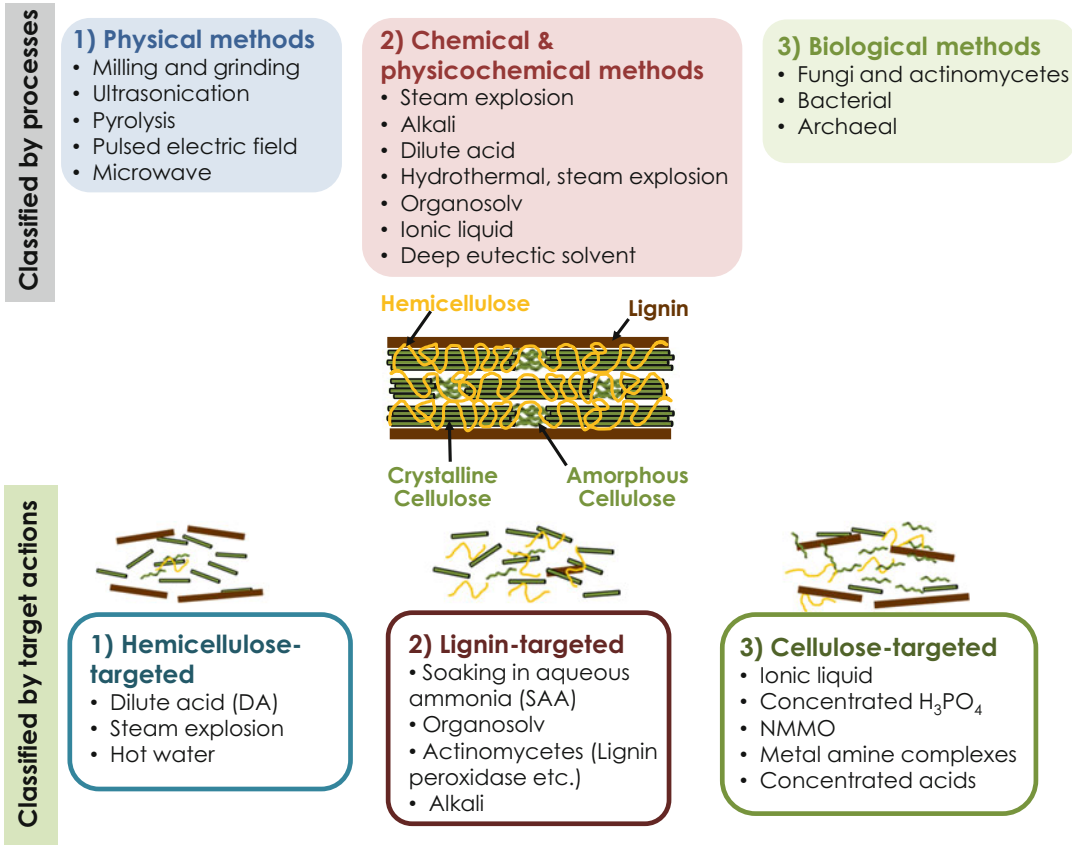


Fig. 2 Classification of pretreatment methods by (1) processes and (2) mode of actions

necessitates a higher cellulase dosage. Second, cellulosic components are embedded in lignin, preventing cellulose accessibility to cellulase (CAC). As a result, most pretreatment technologies have the common goal of increasing cellulose accessibility to cellulase to promote a high enzymatic saccharification rate/yield with minimal enzyme loading. To increase the CAC, researchers have developed various strategies, targeting lignocellulosic components. Three major pretreatment strategies include: (1) removal of hemicelluloses to loosen the chemical structure of lignocellulose [9], (2) lignin removal to unglue the lignocellulosic structure, and (3) decrystallization of crystalline cellulose to improve the hydrolysis rate. Dilute acid, steam explosion, and hot water pretreatments mainly remove the hemicelluloses from lignocellulose. Organosolv and alkaline (Lime, ammonia fiber expansion; AFEX, ammonia recycled percolation; ARP) pretreatments aim to remove lignin, the recalcitrant barrier that causes nonspecific binding of cellulase.

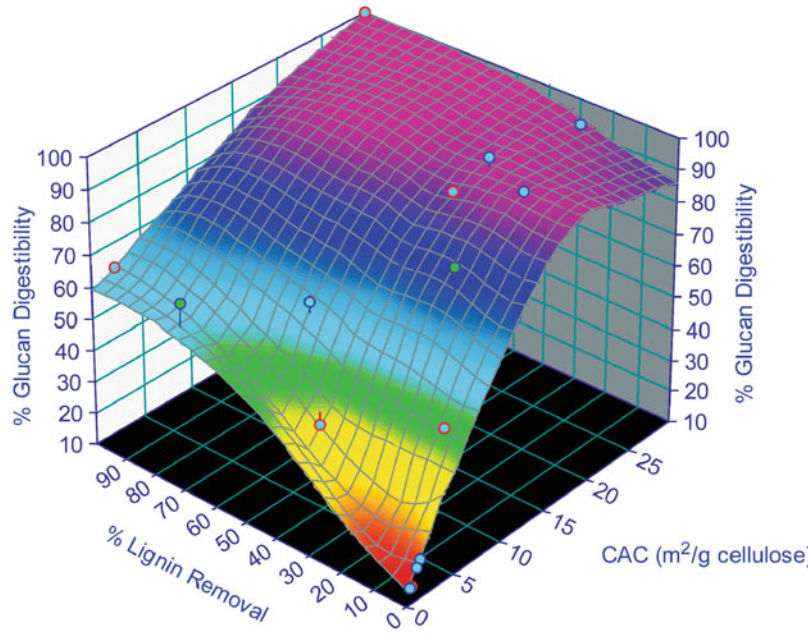
Increasing Cellulose Accessibility to Cellulase (CAC) Is More Important Than Lignin Removal

Two major cell wall components, cellulose and hemicelluloses, are the primary carbohydrate sources for lignocellulose-based bioethanol production by fermentation, whereas cell wall lignin adversely impacts bioconversion [10, 11]. Lignin binds tightly to hemicellulose and cellulose, forming a recalcitrant matrix and creating

physical and chemical barriers to enzymatic hydrolysis. As a physical barrier, lignin blocks the access of hydrolytic enzymes to cellulosic substrates. As a chemical barrier, lignin binds nonspecifically to enzymes by hydrophobic, electrostatic, and hydrogen-bonding interactions, inhibiting the activities of hydrolytic and fermentation enzymes during bioconversion [12–16]. Hence, to facilitate enzymatic hydrolysis, numerous strategies have been developed that use chemical and genetic tools, such as delignification of lignocellulose and genetic modification of plants, to reduce lignin content [17, 18].

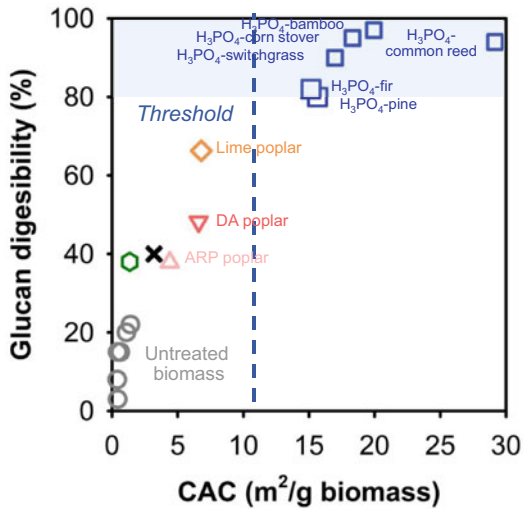
Rollin et al. showed that untreated switchgrass has a low CAC value of <0.5 m^2/g biomass, resulting in a low hydrolysis rate and yield (Fig. 3) [19]. Lignin removal by Soaking in Aqueous Ammonia (SAA) causes a 1.4-fold increase in CAC (from 0.49 to 0.68 m^2/g biomass). Pretreatment with concentrated H_3PO_4 as a cellulose solvent causes a 16-fold increase in CAC (from 0.49 to 8.00 m^2/g biomass), resulting in $>90\%$ hydrolysis yield within 10 h at a low enzyme loading of 3 FPU/g glucan. These results suggest that increasing CAC is more important than lignin removal.

Cellulose solvents constitute a promising pretreatment approach to increase CAC and enhance the saccharification of cellulosic biomass. Solvents, such as ionic liquids, concentrated acids (H_2SO_4 and H_3PO_4), deep eutectic solvents, N-methylmorpholine N-oxide (NMMO), and metal amine complexes, can (partially) dissolve cellulose. Hossain et al. reported that cellulose solvent-based pretreatment with concentrated H_3PO_4 caused a 38-fold increase in CAC of softwoods (from 0.4 to 15.0 m^2/g biomass), resulting in a high hydrolysis yield ($>80\%$) (Fig. 4) [20]. These solvents disrupt intra-/intermolecular hydrogen bonding within cellulose. Upon regeneration in solvents and/or water, the cellulose becomes amorphous [21–23]. Amorphous cellulose has a higher CAC compared with crystalline cellulose, resulting in a high enzymatic hydrolysis rate [24]. Table 1 summarizes the effects of pretreatment methods on the chemical composition and structure of cellulose.



Enhancing Cellulose Accessibility to Cellulase by Ionic Liquid Pretreatment: Interplay Between Cellulose Accessibility and Glucan Digestibility,

Fig. 3 Correlation between cellulose accessibility to cellulase (CAC) and lignin removal with glucan digestibility [19]. (Adapted with permission from the John Wiley and Sons)



Enhancing Cellulose Accessibility to Cellulase by Ionic Liquid Pretreatment: Interplay Between Cellulose Accessibility and Glucan Digestibility, Fig. 4 Correlation of cellulose accessibility to cellulase (CAC) and glucan digestibility in various pretreatment methods [20]

Cellulose Solvent-Based Pretreatment Enhances Cellulose Accessibility to Cellulase

Unlike other cellulose solvents, ionic liquids (ILs) have cations and anions that are important in disrupting intra- and inter-molecular hydrogen bonding of crystalline cellulose to form amorphous cellulose [22, 26, 27]. Table 2 summarizes pretreatment of lignocellulose with various ionic liquids. Amorphous cellulose has a high CAC, making it more susceptible to enzymatic and acid hydrolysis [28]. Moreover, ionic liquids can serve as reaction media for the conversion of dissolved cellulose to value-added products (e.g., 5-HMF, furfural, and levulinic acid) in one-pot with acid catalysts [29–31] or enzymes/microbes [32]. This one-pot approach eliminates the costly separation of sugars.

Pretreatment with cellulose solvents, particularly ionic liquids and concentrated H_3PO_4,

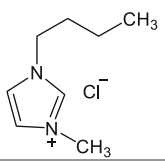
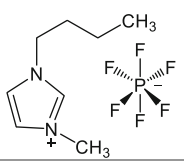
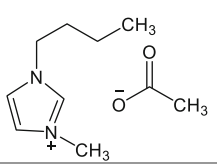
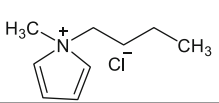
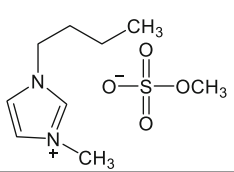
Enhancing Cellulose Accessibility to Cellulase by Ionic Liquid Pretreatment: Interplay Between Cellulose Accessibility and Glucan Digestibility,

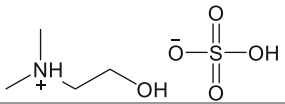
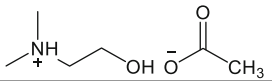
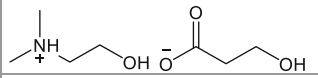
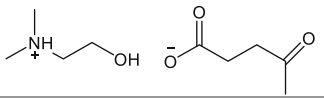
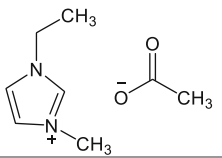
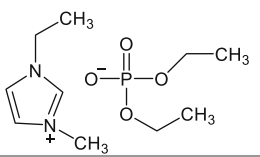
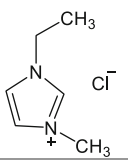
Table 1 Effect of various pretreatments on the chemical composition and structure of lignocellulose [25] with permission from Elsevier

Pretreatment	Increases accessible surface area	Decrystallizes cellulose	Removes hemicellulose	Removes lignin	Alters lignin structure
Ionic liquids/deep eutectic solvents	♦	♦	*	*	*
Concentrated H_3PO_4	♦	♦	♦	*	*
Uncatalyzed steam explosion	♦		♦		*
Liquid hot water	♦	ND	♦		*
pH-controlled hot water	♦	ND	♦		ND
Flow-through liquid hot water	♦	ND	♦	*	*
Dilute acid	♦		♦		♦
Flow-through acid	♦		♦	*	♦
AFEX	♦	♦	*	♦	♦
ARP	♦	♦	*	♦	♦
Lime	♦	ND	*	♦	♦

♦ = major effect, * = minor effect and ND = not determined.

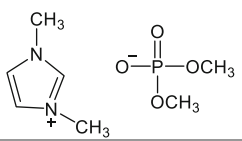
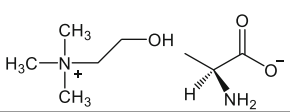
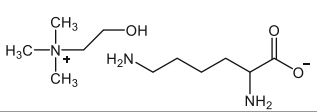
Enhancing Cellulose Accessibility to Cellulase by Ionic Liquid Pretreatment: Interplay Between Cellulose Accessibility and Glucan Digestibility, Table 2 Selected Ionic liquids in lignocellulose pretreatment and enzymatic hydrolysis yield [43]

Ionic liquids	Structure	Feedstock	Pretreatment		Glucan digestibility or TRS (%)	Ref
			Temp (C)	Time (min)		
1-butyl-3-methylimidazolium chloride		Pine	130	1080	97% @ 96 h[a]	[44]
1-butyl-3-methylimidazolium hexafluorophosphate		Corn cob	130	20	33% @ 24 h[b]	[45]
1-butyl-3-methylimidazolium acetate		Bagasse	110	30	96% @ 72 h[a]	[46]
1-butyl-1-methylpyrrolidinium chloride		Corn stover	130	20	76% @ 24 h[b]	[45]
1-butyl-3-methylimidazolium methylsulfate		Corn stover	130	60	15% @ 24 h[a]	[47]

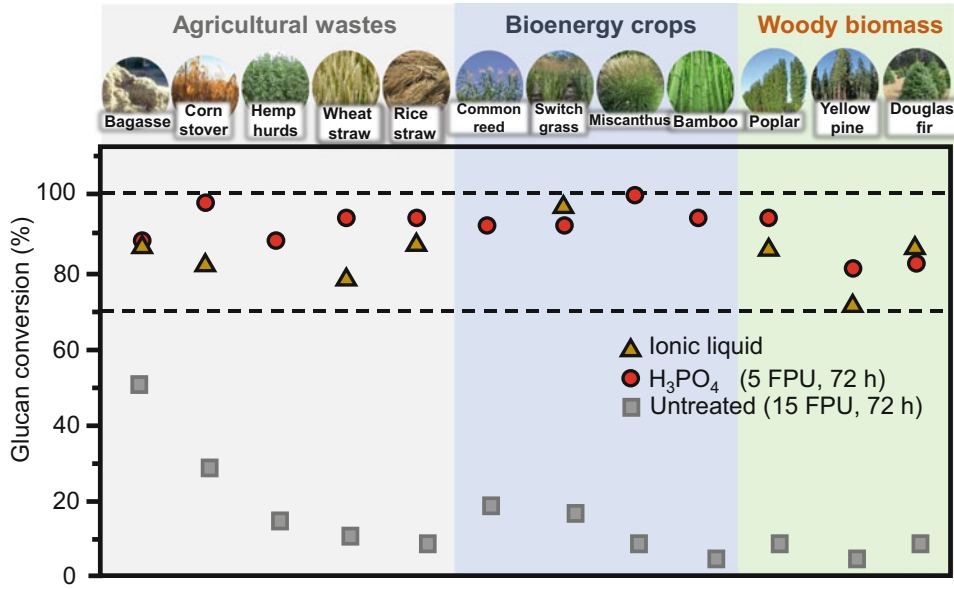
N,N- dimethylethanolamonium hydrogen sulfate		Pine	170	30	75% @ 168 h[a]	[48]
N,N-dimethylethanolamonium acetate		Triticale straw	90	1440	25% @ 11 h[a]	[49]
N,N- dimethylethanolamonium glycolate		Triticale straw	90	1440	28% @ 11 h[a]	[49]
N,N-dimethylethanolammonium succinate		Triticale straw	90	1440	21% @ 11 h[a]	[49]
1-ethyl-3-methylimidazolium acetate		Triticale straw	90	1440	98% @ 11 h[a]	[49]
1-ethyl-3-methylimidazolium diethyl phosphate		Wheat straw	100	60	55% @ 12 h[b]	[50]
1-ethyl-3-methylimidazolium chloride		Rice straw	130	1440	58% @ 72 h[a]	[51]

(continued)

Enhancing Cellulose Accessibility to Cellulase by Ionic Liquid Pretreatment: Interplay Between Cellulose Accessibility and Glucan Digestibility, Table 2 (continued)

Ionic liquids	Structure	Feedstock	Pretreatment		Glucan digestibility or TRS (%)	Ref
			Temp (C)	Time (min)		
1,3-dimethylimidazolium dimethyl phosphate		Bagasse	120	120	70% @ 48 h[b]	[52]
Cholinium glycinate		Wheat straw	90	360	65% @ 48 h[a]	[37]
Cholinium lysinate		Pine	140	60	23% @ 72 h[a]	[53]

Note: [a]glucan digestibility; [b]TRS= total reducing sugar



Enhancing Cellulose Accessibility to Cellulase by Ionic Liquid Pretreatment: Interplay Between Cellulose Accessibility and Glucan Digestibility, Fig. 5 Cellulose solvent-based pretreatments (ionic liquid and H_3PO_4) are feedstock-agnostic [20]. Note: For ionic liquid pretreatment, enzyme loading (FPU/ g glucan) was estimated by its activity (Filter paper unit, FPU) based on glucan content in pretreated lignocellulose [33, 34] as follows: bagasse = 30 [35], Corn stover = 22.2 [36], Wheat straw = 11 [37], rice straw = 35 [38], switchgrass = 31 [39], poplar = 8 [40], pine = 70 [41] and Douglas fir = 14.8 [42]

enhances cellulose accessibility to cellulase (CAC) by breaking the highly ordered hydrogen bonding of crystalline cellulose. Pretreated lignocellulose samples (agricultural wastes, bioenergy crops, and woody biomass) have a high glucan digestibility (>70%) with a low amount of enzyme (5 FPU/g glucan) (Fig. 5). The low amount of costly enzymes required after cellulose solvent-based pretreatments makes this process an economical option in biorefineries.

Cellulose Accessibility to Cellulase Assays: Principles, Applications, and Limitations

The cellulose accessibility to cellulase parameter (CAC) is an important lignocellulose characteristic that affects its hydrolysis rate/yield. The ability to measure the CAC is important to determine the choice of pretreatment, the pretreatment conditions, and severity. Table 3 shows the advantages and disadvantages of selected CAC measurement techniques. Among these techniques, N_2 adsorption/desorption has been widely used to estimate the CAC. However, the small size of N_2 causes the overestimation of cellulose accessibility. This chapter is not intended to provide a prescriptive list of techniques to determine cellulose accessibility. We will focus on the biomimicry technique using a fusion protein that mimics the adsorption of cellulase enzymes on cellulosic substrate.

Biomimicry of a Fusion Protein

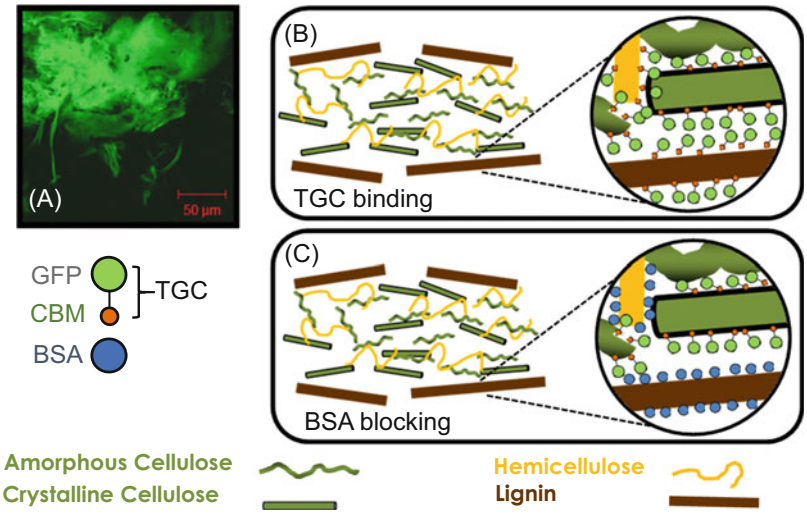
Protein engineering holds great promise in developing new pharmaceutical compounds for medical applications, enhancing proteins for household products, creating efficient enzymes for

Enhancing Cellulose Accessibility to Cellulase by Ionic Liquid Pretreatment: Interplay Between Cellulose Accessibility and Glucan Digestibility, Table 3 Advantages and disadvantages of selected characterization tools for cellulose accessibility measurement

Technique	Advantage	Disadvantage
N_2 adsorption/ desorption [54, 55]	Simple analysis	Time-consuming Application on dry samples. Drying process causes the collapse of pores Small size of N_2 causes overestimation of cellulose accessibility
Mercury porosimetry [56, 57]	Application to samples with a wide range of pore widths, Information of pore size, pore volume distribution, specific area, tortuosity)	Application on dry samples Drying process causes the collapse of pores Inaccurate determination of pore sizes
Solute Exclusion (SE) [58–60]	Simple analysis Application on wet or dry samples	Nonspecific to cellulose Inaccurate determination of absolute pore size and volume distribution Sensitivity to pore shape and osmotic pressure
NMR Cryoporometry [61, 62]	Application on wet samples Recovery of samples because it is a nondestructive technique	Temperature dependence limits determination of pore size range Expensive, long analysis time requires special equipment
Simon's Stain [63–66]	Application on wet samples Short analysis time Application on both interior and exterior surface areas	Sensitivity to pore shape and tortuosity Semi-quantitative
Biomimicry by a fusion protein [23, 67, 68]	Accurate representation of enzyme hydrolysis because the fusion protein is similar in size to cellulase Accurate determination of cellulose accessibility to cellulase	The complexity and time required to express and prepare fusion protein

biorefineries, and in developing bioanalytical tools for surface characterization [69, 70]. Hence, researchers have exploited advances in protein engineering to develop fusion proteins that mimic the properties of cellulase enzymes. Hong et al. developed a quantitative assay to determine CAC with an engineered fusion protein that mimicked cellulase adsorption [67]. The fusion protein, referred to as TGC, contained thioredoxin (*T*), Green Fluorescent Protein (*G*FP), and a family 3 cellulose-binding module (*C*BM3). The ~62 kDa TGC was similar in size to *Trichoderma reesei endo*-glucanase I. The CBM3 module in TGC enabled TGC to adsorb on cellulose [67]. As a result, CAC determination by TGC adsorption is more accurate than conventional methods [68]. The GFP moiety enables TGC to fluoresce under UV excitation. TGC adsorption on the surface of cellulose can be quantitatively measured by the Langmuir adsorption equation with an assumption of monolayer adsorption. CAC values can be calculated by the maximum binding capacity of TGC on cellulose (m^2/g cellulose) or biomass, with the assumption that a molecule of TGC can occupy approximately 21 molecules of cellobiose lattice [71].

Unlike TGC adsorption on pure cellulose, the lignin in lignocellulose can interfere with TGC adsorption because the TGC CBM3 module can bind nonspecifically to lignin. To circumvent this problem, Zhu et al. used bovine serum albumin (BSA) to block nonspecific binding of TGC to the lignin fraction before TGC adsorption (Eq. 1) (Fig. 6) [23]. The total substrate accessibility to cellulase (TSAC) refers to TGC adsorption without BSA blocking (Eq. 2).



Enhancing Cellulose Accessibility to Cellulase by Ionic Liquid Pretreatment: Interplay Between Cellulose Accessibility and Glucan Digestibility,

Fig. 6 Conceptual image of a fusion protein (TGC) adsorbs on lignocellulose surface (**a**) Fluorescence micrographs of lignocellulose (**b**) without BSA blocking and (**c**) with BSA blocking [20]

$$\mathrm{CAC} = \alpha.A_{\max,\mathrm{BSA/TGC}}.N_{\mathrm{A}}.A_{\mathrm{G2}} \qquad (\mathrm{Eq.}(1))$$

where α (21) is the number of cellobiose lattice occupied by a molecule of TGC protein, $A_{\max,\mathrm{BSA/TGC}}$ is the maximum cellulase adsorption capacity (mole cellulase/ g cellulose) after BSA blocking, N_{A} is Avogadro's number ($6.023 \quad 10^{23}$ molecules/mol), A_{G2} is the 110 area of cellobiose lattice ($0.53 \quad 1.04\ \mathrm{nm} = 5.512 \quad 10^{-19}\ \mathrm{m}^2$) [72].

$$\mathrm{TSAC} = \alpha.A_{\max,\mathrm{TGC}}.N_{\mathrm{A}}.A_{\mathrm{G2}} \qquad (\mathrm{Eq.}(2))$$

where $A_{\max,\mathrm{TGC}}$ is the maximum cellulase adsorption capacity (mole cellulase/g cellulose). Non-cellulose accessibility (NCAC) can be determined by the difference between TSAC and CAC (Eq. 3) [20].

$$\mathrm{NCAC} = \mathrm{TSAC} \quad \mathrm{CAC} \qquad (\mathrm{Eq.3})$$

We can engineer different families of CBM and fluorescent probes to distinguish amorphous and crystalline cellulose. Gao et al. engineered a fusion protein (TCC) that contained thioredoxin (*T*), a mono-cherry fluorescent protein (*C*FP), and a family 17 carbohydrate-binding module (*C*BM17) [36]. With the CBM17, TCC could bind only to amorphous cellulose, whereas TGC containing the family 3 carbohydrate-binding module (CBM3) binds nonspecifically to both amorphous and crystalline cellulose. By applying TCC and TGC to cellulosic substrates, one can distinguish amorphous cellulose from crystalline cellulose. Numerous investigators have employed this approach to measure the CAC of untreated and pretreated lignocellulose. They showed that cellulose solvents (ionic liquids and concentrated H_3PO_4) disrupted the highly ordered crystalline cellulose, causing an increase in CAC and high hydrolysis yield/rates [73, 74]. This dual-fusion protein approach enables more accurate determination of CAC compared with other methods, such as N_2 adsorption/desorption, solute exclusion, and Simons' stain method [66]. Nevertheless, this approach requires complex TGC fusion protein expression and purification.

Conclusions and Perspectives

Lignocellulose is a potential feedstock for production of biofuels and bioproducts. Due to its physical recalcitrance, pretreatment is required to enhance cellulose accessibility to cellulase. Ionic liquids are promising compounds for lignocellulose pretreatment. The synergistic effect between cations and anions of an ionic liquid disrupts hydrogen bonding among cellulose chains, increasing cellulose accessibility to cellulase and resulting in high sugar yield. Hence, ionic liquid pretreatment may affect lignin's chemical

structure and its further upgradability. Although ionic liquid pretreatment shows great promise in lignocellulose conversion, several challenges need to be addressed: (1) creating cost-effective ionic liquids (or other cellulose solvents); (2) increasing product titer to reduce product separation cost; (3) decreasing waste generated during cellulose solvent pretreatment; (4) developing spectroscopic tools to identify and quantify changes in lignin's chemical structure that could affect its co-product utilization; (5) exploring new chemistries for carbohydrates/sugar conversion in ionic liquids to avoid costly separation steps; (6) developing efficient approaches for cellulose solvent recycling; and (7) developing techno-economic analysis (TEA) models and life cycle analyses (LCA) of ionic liquid pretreatment for biorefineries.

References

1. DOE: replacing the whole barrel to reduce U.S. dependence on oil. Bioenergy Technologies Office (2013)
2. DECC: UK bioenergy strategy. Department of Energy & Climate Change (2012)
3. Michelin M, Ruiz HA, Silva DP, Ruzene DS, Teixeira JA, Polizeli MLTM (2015) Cellulose from lignocellulosic waste. In: Ramawat KG, Mérillon J-M (eds) Polysaccharides: bioactivity and biotechnology. Springer International Publishing, Cham, pp 475–511
4. Lynd LR, Wyman CE, Gerngross TU (1999) Biocommodity engineering. Biotechnol Prog 15(5): 777–793. https://doi.org/10.1021/bp990109e
5. Ooshima H, Burns DS, Converse AO (1990) Adsorption of cellulase from Trichoderma reesei on cellulose and lignacious residue in wood pretreated by dilute sulfuric acid with explosive decompression. Biotechnol Bioeng 36(5):446–452. https://doi.org/10.1002/bit.260360503
6. Ooshima H, Sakata M, Harano Y (1983) Adsorption of cellulase from Trichoderma viride on cellulose. Biotechnol Bioeng 25(12):3103–3114. https://doi.org/10.1002/bit.260251223
7. Sinitsyn AP, Gusakov AV, Vlasenko EY (1991) Effect of structural and physico-chemical features of cellulosic substrates on the efficiency of enzymatic hydrolysis. Appl Biochem Biotechnol 30(1):43–59. https://doi.org/10.1007/bf02922023
8. Jeoh T, Ishizawa CI, Davis MF, Himmel ME, Adney WS, Johnson DK (2007) Cellulase digestibility of pretreated biomass is limited by cellulose accessibility. Biotechnol Bioeng 98(1):112–122. https://doi.org/10.1002/bit.21408
9. Silveira RL, Stoyanov SR, Gusarov S, Skaf MS, Kovalenko A (2013) Plant biomass recalcitrance: effect of hemicellulose composition on nanoscale forces that control cell wall strength. J Am Chem Soc 135(51):19048–19051. https://doi.org/10.1021/ja405634k
10. Chapple C, Ladisch M, Meilan R (2007) Loosening lignin's grip on biofuel production. Nat Biotechnol 25(7):746–748
11. Chen F, Dixon RA (2007) Lignin modification improves fermentable sugar yields for biofuel production. Nat Biotechnol 25(7):759–761
12. Keating J, Panganiban C, Mansfield S (2006) Tolerance and adaptation of ethanologenic yeasts to lignocellulosic inhibitory compounds. Biotechnol Bioeng 93(6):1196–1206
13. Endo A, Nakamura T, Ando A, Tokuyasu K, Shima J (2008) Genome-wide screening of the genes required for tolerance to vanillin, which is a potential inhibitor of bioethanol fermentation, in Saccharomyces cerevisiae. Biotechnol Biofuels 1(1):3
14. Abramson M, Shoseyov O, Shani Z (2010) Plant cell wall reconstruction toward improved lignocellulosic production and processability. Plant Sci 178(2):61–72
15. Nakagame S, Chandra R, Saddler J (2011) The influence of lignin on the enzymatic hydrolysis of pretreated biomass substrates. In: Sustainable production of fuels, chemicals, and fibers from forest biomass. American Chemical Society, Washington, DC, pp 145–167
16. Pan X (2008) Role of functional groups in lignin inhibition of enzymatic hydrolysis of cellulose to glucose. J Biobaased Mater Bioenergy 2(1):25–32
17. Eudes A, Sathitsuksanoh N, Baidoo E, George A, Liang Y, Yang F, Singh S, Keasling J, Simmons B, Loqué D (2015) Expression of a bacterial 3-dehydroshikimate dehydratase reduces lignin content and improves biomass saccharification efficiency. Plant Biotechnol J 13(9):1241–1250
18. Xu B, Escamilla-Treviño L, Sathitsuksanoh N, Shen Z, Shen H, Percival Zhang YH, Dixon R, Zhao B (2011) Silencing of 4-coumarate: coenzyme A ligase in switchgrass leads to reduced lignin content and improved fermentable sugar yields for biofuel production. New Phytol 192(3):611–625
19. Rollin JA, Zhu Z, Sathitsuksanoh N, Zhang YHP (2011) Increasing cellulose accessibility is more important than removing lignin: a comparison of cellulose solvent-based lignocellulose fractionation and soaking in aqueous ammonia. Biotechnol Bioeng 108(1):22–30. https://doi.org/10.1002/bit.22919
20. Hossain MA, Rahaman MS, Lee D, Phung TK, Canlas CG, Simmons BA, Renneckar S, Reynolds WT, George A, Tulaphol S, Sathitsuksanoh N (2019) Enhanced softwood cellulose accessibility by H3PO4 pretreatment: high sugar yield without compromising

lignin integrity. Ind Eng Chem Res. https://doi.org/10.1021/acs.iecr.9b05873
21. Sathitsuksanoh N, Zhu Z, Wi S, Zhang YHP (2011) Cellulose solvent-based biomass pretreatment breaks highly ordered hydrogen bonds in cellulose fibers of switchgrass. Biotechnol Bioeng 108(3): 521–529. https://doi.org/10.1002/bit.22964
22. Sathitsuksanoh N, Zhu Z, Zhang YHP (2012) Cellulose solvent-based pretreatment for corn stover and avicel: concentrated phosphoric acid versus ionic liquid [BMIM]Cl. Cellulose 19(4):1161–1172. https://doi.org/10.1007/s10570-012-9719-z
23. Zhu Z, Sathitsuksanoh N, Vinzant T, Schell DJ, McMillan JD, Zhang YHP (2009) Comparative study of corn stover pretreated by dilute acid and cellulose solvent-based lignocellulose fractionation: enzymatic hydrolysis, supramolecular structure, and substrate accessibility. Biotechnol Bioeng 103(4):715–724. https://doi.org/10.1002/bit.22307
24. Satari B, Karimi K, Kumar R (2019) Cellulose solvent-based pretreatment for enhanced second-generation biofuel production: a review. Sustain Energy Fuels 3(1):11–62. https://doi.org/10.1039/C8SE00287H
25. Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96(6):673–686. https://doi.org/10.1016/j.biortech.2004.06.025
26. Salapa I, Katsimpouras C, Topakas E, Sidiras D (2017) Organosolv pretreatment of wheat straw for efficient ethanol production using various solvents. Biomass Bioenergy 100:10–16. https://doi.org/10.1016/j.biombioe.2017.03.011
27. Sun N, Rodríguez H, Rahman M, Rogers RD (2011) Where are ionic liquid strategies most suited in the pursuit of chemicals and energy from lignocellulosic biomass? Chem Commun 47(5):1405–1421. https://doi.org/10.1039/C0CC03990J
28. Bertran MS, Dale BE (1985) Enzymatic hydrolysis and recrystallization behavior of initially amorphous cellulose. Biotechnol Bioeng 27(2):177–181. https://doi.org/10.1002/bit.260270212
29. Tulaphol S, Hossain MA, Rahaman MS, Liu LY, Phung TK, Renneckar S, Grisdanurak N, Sathitsuksanoh N (2019) Direct production of levulinic acid in one pot from hemp hurd by dilute acid in ionic liquids. Energy Fuel. https://doi.org/10.1021/acs.energyfuels.9b03134
30. Liu L, Yang X, Hou Q, Zhang S, Ju M (2018) Corn stalk conversion into 5-hydroxymethylfurfural by modified biochar catalysis in a multi-functional solvent. J Clean Prod 187:380–389. https://doi.org/10.1016/j.jclepro.2018.03.234
31. Alam MI, De S, Khan TS, Haider MA, Saha B (2018) Acid functionalized ionic liquid catalyzed transformation of non-food biomass into platform chemical and fuel additive. Ind Crop Prod 123:629–637. https://doi.org/10.1016/j.indcrop.2018.07.036
32. Shi J, Gladden JM, Sathitsuksanoh N, Kambam P, Sandoval L, Mitra D, Zhang S, George A, Singer SW, Simmons BA, Singh S (2013) One-pot ionic liquid pretreatment and saccharification of switchgrass. Green Chem 15(9):2579–2589. https://doi.org/10.1039/C3GC40545A
33. Brummer V, Skryja P, Jurena T, Hlavacek V, Stehlik P (2014) Suitable technological conditions for enzymatic hydrolysis of waste paper by Novozymes® enzymes NS50013 and NS50010. Appl Biochem Biotechnol 174(4):1299–1308. https://doi.org/10.1007/s12010-014-1119-4
34. Cannella D, Hsieh CWC, Felby C, Jørgensen H (2012) Production and effect of aldonic acids during enzymatic hydrolysis of lignocellulose at high dry matter content. Biotechnol Biofuels 5(1):26. https://doi.org/10.1186/1754-6834-5-26
35. Qiu Z, Aita GM, Walker MS (2012) Effect of ionic liquid pretreatment on the chemical composition, structure and enzymatic hydrolysis of energy cane bagasse. Bioresour Technol 117:251–256. https://doi.org/10.1016/j.biortech.2012.04.070
36. Gao X, Kumar R, Singh S, Simmons BA, Balan V, Dale BE, Wyman CE (2014) Comparison of enzymatic reactivity of corn stover solids prepared by dilute acid, AFEX™, and ionic liquid pretreatments. Biotechnol Biofuels 7(1):71. https://doi.org/10.1186/1754-6834-7-71
37. Ren H, Zong MH, Wu H, Li N (2016) Efficient pretreatment of wheat straw using novel renewable cholinium ionic liquids to improve enzymatic saccharification. Ind Eng Chem Res 55(6):1788–1795. https://doi.org/10.1021/acs.iecr.5b03729
38. Hou XD, Li N, Zong MH (2013) Significantly enhancing enzymatic hydrolysis of rice straw after pretreatment using renewable ionic liquid–water mixtures. Bioresour Technol 136:469–474. https://doi.org/10.1016/j.biortech.2013.02.118
39. Li C, Knierim B, Manisseri C, Arora R, Scheller HV, Auer M, Vogel KP, Simmons BA, Singh S (2010) Comparison of dilute acid and ionic liquid pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. Bioresour Technol 101(13):4900–4906. https://doi.org/10.1016/j.biortech.2009.10.066
40. Wu L, Kumagai A, Lee SH, Endo T (2014) Synergistic effect of delignification and treatment with the ionic liquid 1-ethyl-3-methylimidazolium acetate on enzymatic digestibility of poplar wood. Bioresour Technol 162:207–212. https://doi.org/10.1016/j.biortech.2014.03.144
41. Li C, Sun L, Simmons BA, Singh S (2013) Comparing the recalcitrance of eucalyptus, pine, and switchgrass using ionic liquid and dilute acid pretreatments. Bioenergy Res 6(1):14–23. https://doi.org/10.1007/s12155-012-9220-4
42. Socha AM, Plummer SP, Stavila V, Simmons BA, Singh S (2013) Comparison of sugar content for ionic liquid pretreated Douglas-fir woodchips and

forestry residues. Biotechnol Biofuels 6(1):61. https://doi.org/10.1186/1754-6834-6-61
43. Sathitsuksanoh N, George A, Zhang YHP (2013) New lignocellulose pretreatments using cellulose solvents: a review. J Chem Technol Biotechnol 88(2):169–180. https://doi.org/10.1002/jctb.3959
44. Trinh LTP, Lee YJ, Lee JW, Lee HJ (2015) Characterization of ionic liquid pretreatment and the bioconversion of pretreated mixed softwood biomass. Biomass Bioenergy 81:1–8. https://doi.org/10.1016/j.biombioe.2015.05.005
45. Li Q, Jiang X, He Y, Li L, Xian M, Yang J (2010) Evaluation of the biocompatibile ionic liquid 1-methyl-3-methylimidazolium dimethylphosphite pretreatment of corn cob for improved saccharification. Appl Microbiol Biotechnol 87(1):117–126. https://doi.org/10.1007/s00253-010-2484-8
46. Hashmi M, Sun Q, Tao J, Wells T, Shah AA, Labbé N, Ragauskas AJ (2017) Comparison of autohydrolysis and ionic liquid 1-butyl-3-methylimidazolium acetate pretreatment to enhance enzymatic hydrolysis of sugarcane bagasse. Bioresour Technol 224:714–720. https://doi.org/10.1016/j.biortech.2016.10.089
47. Wu H, Mora-Pale M, Miao J, Doherty TV, Linhardt RJ, Dordick JS (2011) Facile pretreatment of lignocellulosic biomass at high loadings in room temperature ionic liquids. Biotechnol Bioeng 108(12):2865–2875. https://doi.org/10.1002/bit.23266
48. Gschwend FJV, Chambon CL, Biedka M, Brandt-Talbot A, Fennell PS, Hallett JP (2019) Quantitative glucose release from softwood after pretreatment with low-cost ionic liquids. Green Chem 21(3):692–703. https://doi.org/10.1039/C8GC02155D
49. Fu D, Mazza G, Tamaki Y (2010) Lignin extraction from straw by ionic liquids and enzymatic hydrolysis of the cellulosic residues. J Agric Food Chem 58(5):2915–2922. https://doi.org/10.1021/jf903616y
50. Li Q, He YC, Xian M, Jun G, Xu X, Yang JM, Li LZ (2009) Improving enzymatic hydrolysis of wheat straw using ionic liquid 1-ethyl-3-methyl imidazolium diethyl phosphate pretreatment. Bioresour Technol 100(14):3570–3575. https://doi.org/10.1016/j.biortech.2009.02.040
51. Nguyen TAD, Kim KR, Han SJ, Cho HY, Kim JW, Park SM, Park JC, Sim SJ (2010) Pretreatment of rice straw with ammonia and ionic liquid for lignocellulose conversion to fermentable sugars. Bioresour Technol 101(19):7432–7438. https://doi.org/10.1016/j.biortech.2010.04.053
52. Bahrani S, Raeissi S, Sarshar M (2015) Experimental investigation of ionic liquid pretreatment of sugarcane bagasse with 1,3-dimethylimadazolium dimethyl phosphate. Bioresour Technol 185:411–415. https://doi.org/10.1016/j.biortech.2015.02.085
53. Dutta T, Papa G, Wang E, Sun J, Isern NG, Cort JR, Simmons BA, Singh S (2018) Characterization of lignin streams during bionic liquid-based pretreatment from grass, hardwood, and softwood. ACS Sustain Chem Eng 6(3):3079–3090. https://doi.org/10.1021/acssuschemeng.7b02991
54. Wiman M, Dienes D, Hansen MAT, Meulen VDT, Zacchi G, Lidén G (2012) Cellulose accessibility determines the rate of enzymatic hydrolysis of steam-pretreated spruce. Bioresour Technol 126:208–215. https://doi.org/10.1016/j.biortech.2012.08.082
55. Li C, Cheng G, Balan V, Kent MS, Ong M, Chundawat SPS, Sousa Ld, Melnichenko YB, Dale BE, Simmons BA, Singh S (2011) Influence of physico-chemical changes on enzymatic digestibility of ionic liquid and AFEX pretreated corn stover. Bioresour Technol 102(13):6928–6936. https://doi.org/10.1016/j.biortech.2011.04.005
56. Vitas S, Segmehl JS, Burgert I, Cabane E (2019) Porosity and pore size distribution of native and delignified beech wood determined by mercury intrusion porosimetry. Materials (Basel) 12(3):416. https://doi.org/10.3390/ma12030416
57. Giesche H (2006) Mercury porosimetry: a general (practical) overview. Part Part Syst Charact 23(1):9–19. https://doi.org/10.1002/ppsc.200601009
58. Beecher JF, Hunt CG, Zhu JY (2009) Tools for the characterization of biomass at the nanometer scale. In: The nanoscience and technology of renewable biomaterials, Wiley-Blackwell, Hoboken, New Jersey, pp 61–90
59. Stone JE, Scallan AM, Donefer E, Ahlgren E (1969) Digestibility as a simple function of a molecule of similar size to a cellulase enzyme. In: Cellulases and their applications, vol. 95. Advances in Chemistry, vol. 95. American Chemical Society, Washington, DC, pp 219–241
60. Tarkow H, Feist WC, Southerland CF (1966) Interaction of wood with polymeric materials. Penetration versus molecular size. Forest Prod J 16(10):61–65
61. Mitchell J, Webber JBW, Strange JH (2008) Nuclear magnetic resonance cryoporometry. Phys Rep 461(1):1–36. https://doi.org/10.1016/j.physrep.2008.02.001
62. Meng X, Foston M, Leisen J, DeMartini J, Wyman CE, Ragauskas AJ (2013) Determination of porosity of lignocellulosic biomass before and after pretreatment by using Simons' stain and NMR techniques. Bioresour Technol 144:467–476. https://doi.org/10.1016/j.biortech.2013.06.091
63. Simons FL (1950) A stain for use in the microscopy of beaten fibers. Tappi 33(7):312–314
64. Arantes V, Saddler JN (2011) Cellulose accessibility limits the effectiveness of minimum cellulase loading on the efficient hydrolysis of pretreated lignocellulosic substrates. Biotechnol Biofuels 4(1):3. https://doi.org/10.1186/1754-6834-4-3
65. Chandra R, Ewanick S, Hsieh C, Saddler JN (2008) The characterization of pretreated lignocellulosic substrates prior to enzymatic hydrolysis, part 1: a modified Simons' staining technique. Biotechnol Prog 24(5):1178–1185. https://doi.org/10.1002/btpr.33
66. Esteghlalian AR, Bilodeau M, Mansfield SD, Saddler JN (2001) Do enzymatic hydrolyzability and Simons'

stain reflect the changes in the accessibility of lignocellulosic substrates to cellulase enzymes? Biotechnol Prog 17(6):1049–1054. https://doi.org/10.1021/bp0101177
67. Hong J, Ye X, Zhang YHP (2007) Quantitative determination of cellulose accessibility to cellulase based on adsorption of a nonhydrolytic fusion protein containing CBM and GFP with its applications. Langmuir 23(25):12535–12540. https://doi.org/10.1021/la7025686
68. Zhang YHP, Lynd LR (2004) Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnol Bioeng 88:797–824
69. Woodley JM (2013) Protein engineering of enzymes for process applications. Curr Opin Chem Biol 17(2):310–316. https://doi.org/10.1016/j.cbpa.2013.03.017
70. Walsh G (2003) Pharmaceutical biotechnology products approved within the European Union. Eur J Pharm Biopharm 55(1):3–10. https://doi.org/10.1016/S0939-6411(02)00165-0
71. Hong J, Ye X, Wang Y, Zhang YHP (2008) Bioseparation of recombinant cellulose-binding module-proteins by affinity adsorption on an ultra-high-capacity cellulosic adsorbent. Anal Chim Acta 621(2):193–199
72. Gardner KH, Blackwell J (1974) The structure of native cellulose. Biopolymers 13(10):1975–2001. https://doi.org/10.1002/bip.1974.360131005
73. Kuo CH, Lee CK (2009) Enhancement of enzymatic saccharification of cellulose by cellulose dissolution pretreatments. Carbohydr Polym 77(1):41–46. https://doi.org/10.1016/j.carbpol.2008.12.003
74. Dadi AP, Varanasi S, Schall CA (2006) Enhancement of cellulose saccharification kinetics using an ionic liquid pretreatment step. Biotechnol Bioeng 95(5):904–910. https://doi.org/10.1002/bit.21047