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Physical and Chemical Features of Pretreated Biomass that Influence Macro-/Micro-accessibility and Biological Processing

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14.1 Introduction

Lignocellulosic biomass containing about 55–65 wt% sugars in the form of polymeric structural carbohydrates can be a sustainable feedstock for production of a variety of sugars that can in turn be converted into fuels and chemicals [1–3]. For biological deconstruction of structural carbohydrates to sugars, tiny protein molecules called enzymes need to reach appropriate substrates to depolymerize them. In native plants however, carbohydrates are trapped in a complex matrix comprising non-sugar constituents and polymers such as lignin, which forms a strong and complex sheath around carbohydrates and presents challenges to effective and economical release of sugars from biomass [1,2,4,5]. Prior to biological conversion, some kind of treatment of biomass is therefore needed to make the carbohydrates accessible [6,7]. This pretreatment can be purely mechanical, thermal, chemical, biological, or combinations of these, as reviewed elsewhere [8–10]. Thermochemical pretreatments, however, are considered leading options due to their comparatively short reaction time, effectiveness, and lower energy requirements compared to mechanical options [11–13]. Nonetheless, thermochemical pretreatments typically need

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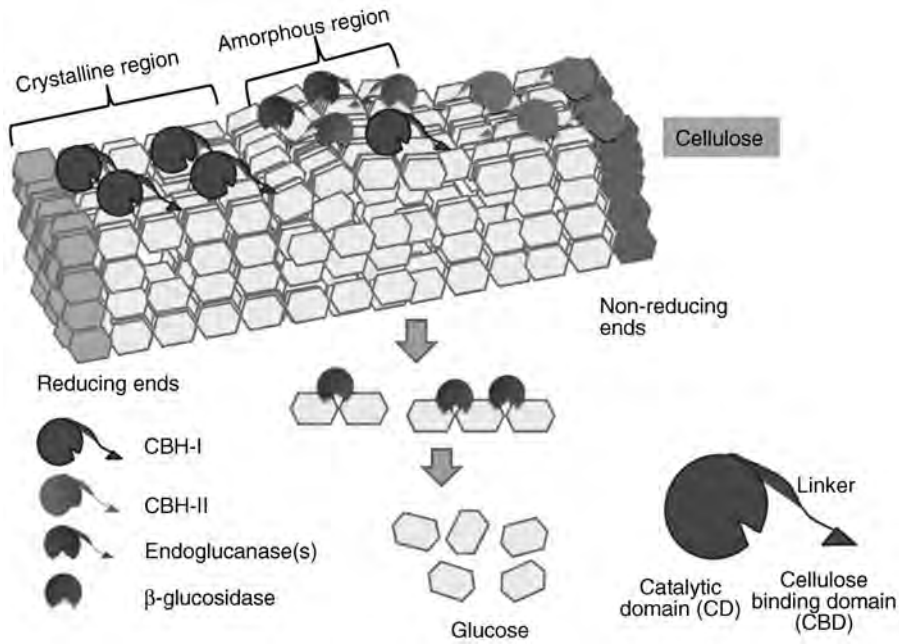

Figure 14.1 *A simplified schematic of cellulose hydrolysis by a typical non-complexed cellulase system mediated by cellobiohydrolases (CBH I and CBH II) and endoglucanases (EG I to EG V) adsorption, cellobiose and cellodextrin production, and their catalysis to glucose by β-glucosidase. Also shown is one of the cellulase components with different domains.*

some mechanical treatment such as size reduction prior to or after pretreatment to realize acceptable performance [14,15].

Enzymatic conversion (saccharification/hydrolysis) of structural carbohydrates is a heterogeneous phenomenon which, unlike homogeneous reactions, involves two major steps: enzyme adsorption on the substrate surface and hydrolysis of the polymers to form shorter chained molecules [16–18], as depicted in Figure 14.1. Compared to the hydrolysis step, enzyme adsorption is fast and usually completed within a few hours (*c.* 2 hours compared to 72–120 hours for hydrolysis, as shown in Figure 14.2) [18,19]. Most carbohydrases, at least in the fungi family that includes cellulases and hemicellulases, are composed of two distinct domains connected by a peptide linker: a cellulose or carbohydrate binding domain (CBM) and a catalytic domain (CD) [20,21], schematically shown in Figure 14.1. Although adsorption is largely mediated by CBMs, catalytic domains also participate in adsorption [22–25].

The literature suggest that several physical and chemical features of pretreated biomass impact its biological conversion including surface area/porosity; hemicellulose and lignin contents; particle size; and cellulose crystallinity, type, and chain length [18,26–28]. However, we believe that all these features are aspects, directly or indirectly, of two main factors that most control conversion: (1) enzyme accessibility to their appropriate substrates; and (2) enzyme effectiveness once adsorbed on the surface. Because a few cellulolytic and xylanolytic enzymes such as β-glucosidase and β-xylosidase catalyze homogenous reactions [29,30], some of the above-mentioned features do not seem to play any direct role in controlling accessibility of these enzymes to their substrates to the best of our knowledge. Nevertheless, they can affect their effectiveness [31–33]. Enzyme accessibility can be further categorized into macro- and micro-accessibility. In this chapter, the impact of physical and chemical features on cellulase macro-

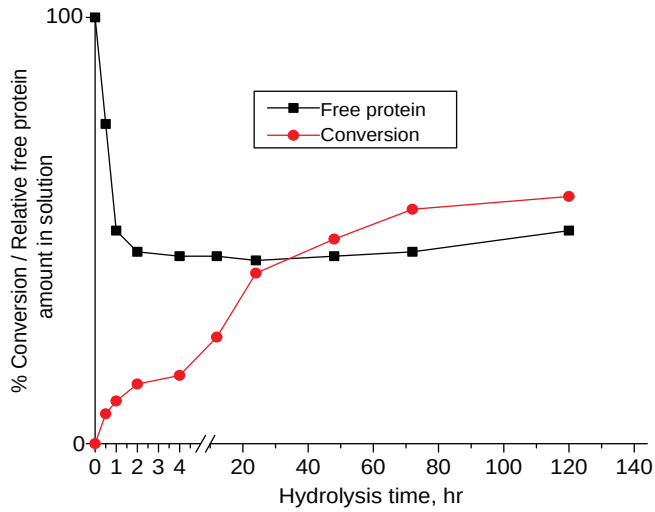

Figure 14.2 *A typical profile of cellulose hydrolysis progression and relative amounts of free protein in solution over hydrolysis time.*

and micro-accessibility and enzyme effectiveness once adsorbed (or in the solution for enzymes which do not participate in adsorption) are reviewed.

14.2 Definitions of Macro-/Micro-accessibility and Effectiveness

Except for a few soluble lower-molecular-weight glucose oligomers such as cellobiose and higher cellodextrins [34], enzymatic hydrolysis of cellulose is a heterogeneous reaction that requires enzymes to adsorb onto a cellulose surface with specific/common binding sites [17,35–38]. The availability of cellulose binding sites to cellulase is called accessibility, and accessibility has been defined both in comparative and quantitative terms. The quantitative definition is often expressed in terms of the maximum adsorption capacity (σ_{max} in μg or mg/g biomass) of cellulase (or its individual components) per gram of biomass or glucan and can be estimated by fitting multiple points of cellulase adsorption data to a non-linear Langmuir model [16–19]. Adsorption data is commonly collected by equilibrating various concentrations of enzymes with a given mass of biomass at non-hydrolytic temperatures, for example 4 °C, to minimize reaction during the measurement [36,39]. The comparative definition is often used to represent the change in accessibility and/or relative accessibility by performing adsorption with a single or multiple concentrations of enzymes, with adsorption expressed as μg or mg protein/g biomass (or glucan) [40–42].

Here we postulate a new concept based on dividing accessibility, at least for real biomass, into two stages: macro and micro. Although techniques to quantify the relative role of each in governing overall accessibility are not yet available due to the complexity of cellulosic biomass, pure cellulose (e.g., Avicel) can be pictured as being completely macro-accessible; it has no obvious obstacles (such as lignin and hemicelluloses) to prevent cellulase enzymes from reaching the cellulose surface, provided physiochemical conditions such as solids concentration and pH are optimized to not impede accessibility [16,43,44]. On the other hand, the hemicellulose/lignin sheath in cellulosic biomass interferes with cellulase reaching cellulose, limiting the ability for enzymes to attach to cellulose [4,45,46]. Carbohydrates in untreated and

pretreated real biomass can therefore be pictured as having varying degrees of macro-accessibility compared to pure cellulose or hemicelluloses, depending on the amount and nature of residual lignin and/or hemicellulose. Once enzymes reach the cellulose surface, a second stage of accessibility (micro-accessibility) can be pictured in terms of how readily the enzymes can reach cellulose binding/catalytic sites buried within the compact, semi-crystalline cellulose structure. Amorphous cellulose can be thought of as representing the highest degree of macro- and micro-accessibility in that cellulase enzymes should be able to reach all cellulose chains. From the cell-wall physiology point of view, cellulase access to cellulose microfibrils and elementary fibrils within cellulose microfibers, respectively, can be referred to as macro- and micro-accessible. Emergence of new tools to measure cellulase–cellulose interactions, such as that developed by Hong *et al.* [39], would be very valuable in clarifying how macro- and micro-accessibility influence cellulose hydrolysis.

Enzyme effectiveness can be defined by the increase in hydrolysis rate/yields with little or no change in accessibility quantified by either the maximum adsorption capacity (MAC), estimated by non-linear fit of adsorption data to the Langmuir model, or the change in protein adsorption per unit biomass. For example, although somewhat contentious, temperature is believed to have limited impact on enzyme access to substrate as shown in the literature by a negligible change in enzyme adsorption with temperature [47]. However, temperature has a great impact on enzyme effectiveness. As another example, most inhibitors such as cellobiose and xylan oligomers have been shown to greatly impact enzyme effectiveness [48–52], but limited studies have indicated limited impact on accessibility. However, Kumar and Wyman showed that sugars, and especially cellobiose, can cause enzyme desorption even at low concentrations [36], a result substantiated in a study by Kristensen *et al.* [53].

14.3 Features Influencing Macro-accessibility and their Impacts on Enzyme Effectiveness

14.3.1 Lignin

Role in Accessibility

Based on limited data, lignin removal does not appear to make morphological changes in the cellulose structure such as cellulose chain length [28,54,55] or cellulose crystallinity; the effects on cellulose macro-accessibility therefore appear more important than micro-accessibility. In plants, the amounts of p-hydroxyphenyl (*H*), guaiacyl (*G*), and syringyl (*S*) lignin varies with species and cell wall, and the secondary cell wall is generally found to be more lignified than the primary cell wall [56–58]. Woody biomass, dicots, and especially softwoods generally have higher lignin content than agricultural residues and herbaceous energy crops, that is, monocots [59–61]. Unfortunately, conventional analysis as acid-soluble and acid-insoluble (Klason) lignin by gravimetric or other methods does not distinguish the impact of lignin from individual cell walls on cellulose digestibility, limiting knowledge of its role.

Lignin appears to be a major impediment to cellulose accessibility and ultimately to polysaccharide saccharification, as most studies in the literature reported that enzymatic conversion of polysaccharides is enhanced by down-regulation of lignin in genetically modified plants and/or delignification of hardwood/softwood and other lignocellulosics [61–70]. Others have found no correlation or a negative correlation between lignin content/removal and cellulose digestibility [4,41,71–74] but, in most of these studies, other effects accompanying delignification or the thermal treatment used for lignin removal makes it difficult to interpret the outcomes.

Although removing lignin appears critical to making biomass amenable to enzymatic hydrolysis in some situations, this conclusion may not be universally applicable as some feedstocks with equal or somewhat less lignin than hardwoods or softwoods, such as corn stover, are susceptible to biological catalysis without significant lignin removal. For example, in a collaborative project jointly funded by DOE and USDA, we

found that corn stover solids prepared by ammonia fiber expansion (AFEX) pretreatment that removed little if any lignin were equally or more susceptible to enzymes as solids prepared by pretreatments that removed a significant amount of lignin [75,76]. Although conditions vary with the type of biomass and pretreatment catalyst type and loading, most leading pretreatments were effective in the range of 140–200 °C; soaking in aqueous ammonia (SAA) and lime were exceptions that were quite effective at lower temperatures [77–79]. These temperatures are well above the glass transition temperature (T_g) of lignin of 80–130 °C, recognizing that the latter depends on the lignin state and measurement methods [80,81]. However, some plasticizers such as glycerol have been reported to lower the lignin T_g[82]. Therefore, lignin melting and relocation appears to occur during most leading thermochemical pretreatments and seems particularly certain for steam explosion and dilute-acid pretreatments in several studies [46,83–86] and in a recent study for AFEX pretreatment of corn stover [57,87].

The impact of lignin removal from biomass on cellulase accessibility to cellulose is not entirely clear. Based on hypothetical models of the intricate lignin-hemicellulose-cellulose structure presented in the literature, hemicellulose appears to form a sheath around cellulose chains, and lignin in turn surrounds hemicellulose. It can therefore be hypothesized that lignin does not directly restrict cellulase accessibility to cellulose to the extent that hemicellulose does, although access to hemicellulose is strongly limited by lignin. Unfortunately, experimental data to support such hypotheses are scarce.

Several studies have reported that lignin removal affects digestibility of hemicellulose more than that of cellulose [71,88–93]. For example, adding crude cellulase at a loading of 25 IFPU/g (international filter paper units) glucan to hybrid poplar and sugar cane bagasse that had lignin selectively removed by peracetic acid pretreatment resulted in low glucan digestibility, unless supplemented with an industrial-grade xylanase SP431 or SP431 supplemented with Novozyme®188 β-glucosidase [89]. Nevertheless, as recently discovered, this result could be due to strong cellulase inhibition by xylooligomers released during enzymatic hydrolysis, which can be relieved by these supplementary enzymes [50–52,94]. In a recent study with highly purified enzyme components and alkaline-peroxide-delignified corn stover, lignin appeared to have a more direct impact on xylan than glucan accessibility [95]. In another paper at that time, Kumar and Wyman showed that selective removal of lignin from corn stover using peracetic acid did not significantly increase cellulase accessibility to cellulose, as measured by purified Cel7A adsorption. Instead, lignin removal appeared to more directly enhance xylan accessibility, which in turn affected cellulose accessibility, as evidenced by a much higher increase in digestion of xylan than of glucan and a linear relation between the percentage increase in xylan and glucan removal [17,96]. Thus, although lignin may not appear to affect cellulose accessibility directly, lignin removal accelerates xylan removal via both biological and thermal means [97], thereby reducing occlusion of glucan chains by hemicellulose [40,95] and apparently greatly enhancing cellulose macro-accessibility. Lignin removal can also alter other biomass features such as increasing Brunauer–Emmett–Teller (BET) surface area and biomass crystallinity [98,99]. However, more investigation is needed to clarify the role of lignin in cellulose macro-/micro-accessibility.

Role in Enzymes Effectiveness

Leading thermochemical pretreatments result in partial removal and/or dislocation of lignin, and some of the lignin solubilized during pretreatment has been shown to precipitate back on cellulose fibers in droplet form [46,83]. The literature suggests that lignin droplets deposited on cellulose may interact with water, as one study showed that hydrophobic surfaces at a macroscopic level do not repel but attract water [100] to form a boundary layer impeding cellulase movement and limiting cellulose accessibility [46,84,101]. However, the source of these droplets was not possible to determine with the conventional NREL two-step acid hydrolysis method often employed for K-lignin determination, as some hemicellulose during pretreatment degrades to lignin-like compounds called humins (pseudo-lignin, char) and may also deposit onto cellulose

[97,102–104]. Enzymes can also unproductively bind to lignin, and possibly to hemicellulose-derived humins, to limit enzyme effectiveness [33,84,105–112]. Unproductive adsorption of cellulase or other enzymes on lignin is hypothesized to be due to hydrophobic interactions between the two [113–116]. Proteins, and especially cellulase that is highly hydrophobic due to clusters of closely located non-polar residues on its surface [117–121], tend to adsorb strongly on hydrophobic surfaces [100,114]; that attachment results in conformational changes and consequently irreversible adsorption and deactivation [122–125]. In line with this, hydrolysis yields and free enzyme concentrations have been reported to increase with increased cellulase hydrophilicity [122,123].

Lignin can impact performance in other ways. First, lignin linkages with cellulose [126–128] should presumably impede the processive action of enzyme components. In addition, lignin breakdown products generally inhibit fermentation and cellulase effectiveness [84,107,129–134]. Moreover, lignin and its derivatives were also reported to precipitate with proteins [135–137]. Overall, lignin may reduce the amount of active enzyme available for cellulose hydrolysis, but its influence on the effectiveness of adsorbed cellulase needs more study.

14.3.2 Hemicellulose

Role in Accessibility

Based on lignocellulosic biomass data and models in the literature, hemicellulose appears to play a direct and vital role in cellulase accessibility to cellulose. Hemicellulose, the other major carbohydrate in biomass, is composed of combinations of hexose and pentose sugars, namely arabinoxylan, arabinogalactan, glucomannan, galactoglucomannan, and xyloglucan [138,139]. Furthermore, the chains of these hemicellulose compounds are often substituted with uronic and glucuronic acids (one per ten xylopyranosyl residues in hardwoods) and acetate (grasses and agricultural residues) [60,140]. Although it would greatly help in designing better pretreatment and enzyme cocktails, unfortunately the exact amounts of these complex hemicellulose polymers cannot be determined due to the complex structure of biomass and limitations in current invasive/non-invasive compositional analysis tools. Xylan, a polymer of xylose, is the major constituent in hemicellulose (>85%) for most lignocellulosic feedstocks except softwoods, for which mannan dominates. Because hemicellulose removal by thermochemical pretreatments is generally not highly selective, its removal is often accompanied by other changes such as a reduction in the cellulose degree of polymerization (DP), creation/enhancement of nanopores within cellulose fibers, and changes in cross-sectional radius of the crystalline cellulose fibril [28,85,141–143], with these effects heavily impacting cellulose micro-accessibility. However, the scale of these contributions to cellulose micro-accessibility appears to be smaller than the increase in cellulose macro-accessibility; hemicellulose removal is therefore attributed to be mostly responsible for enhancing biomass macro-accessibility.

Hemicellulose has been pictured as obstructing cellulase access to cellulose by forming a sheath around glucan chains [40,94,96,99,144–147], with several studies showing a direct relationship between cellulose digestibility and hemicellulose removal [4,68,71,148–154]. However, as reported by Torget *et al.* for short rotation woody crops (silver maple, sycamore, and black locust) and corn residues (stover and cobs), hemicellulose removal was complete at 140 °C and 160 °C for all these substrates. However, woods and stover solids prepared by dilute acid pretreatment at 160 °C were more digestible than when prepared at 140 °C, suggesting that hemicellulose removal is not the sole factor impacting cellulose conversion [155]. In addition, some reports do not postulate any role for hemicellulose removal in changing cellulose digestibility [156–158]. Unfortunately, as discussed above, hemicellulose alteration during pretreatment also disrupts other biomass components [28,158–162]. Reports on impact of selective hemicellulose removal on

cellulose accessibility are scarce, making it challenging to draw firm conclusions about the degree to which it controls access of enzymes to cellulose.

In addition, some argue that hemicellulose may actually be a marker signaling disruption of the far less soluble lignin and that lignin disruption (and/or lignin-carbohydrate complex) could be the key to greater digestion [68,164–167]. In contrast to traditional beliefs, AFEX pretreatment (which was earlier believed to enhance digestibility without removing any components) has recently been shown to solubilize hemicellulose as oligomers that deposited back onto the biomass due to ammonia evaporation, thus enhancing cellulose accessibility [38,57].

Jeoh *et al*. [4,40] reported that cellulose accessibility increased with xylan removal by dilute acid pretreatment of corn stover, as measured by the adsorption of fluorescent labeled Cel7A (CBHI). Furthermore, several reports showed significant effects of xylanase supplementation to cellulase on digestion of glucan as well as xylan, and linear relationships between xylan and glucan digestion suggested that xylan removal affects cellulose accessibility [94,96,145,147,168–171]. Although further investigation is needed, a recent study showed that solubilized hemicellulose oligomers, which can make up a large percentage of the solubles produced by enzymatic hydrolysis and pretreatment for lower enzymes loadings and some pretreatments [50], can negatively impact cellulose accessibility through reduced cellulase adsorption [172].

The xylan backbone of hemicellulose is often substituted with uronic (methyl) and glucuronic acids (up to 6 wt% of dry biomass, and collectively termed glucuronoxylan), acetate (up to 5 wt% of dry biomass), and/or arabinose (up to 3.5 wt% of dry biomass, collectively termed arabinoxylan). Although xylan removal has been reported to affect cellulose accessibility in several studies, the effect of side-chain removal on enzymatic hydrolysis has received little attention. In particular, almost no studies have been directed at thermochemical removal/modification of arabinose; this is hypothesized to be directly ester-bonded with lignin and to significantly affect lignin-carbohydrate complex (LCC) linkages, thus enhancing accessibility and enzymes effectiveness. In recent studies, however, it has been shown that arabinose side-chain modification is one of the reasons why AFEX pretreatment is more effective for grasses than other kinds of biomass [60]. Other side-chain components found in almost all types of biomass are as the anhydrous form of acetic acid called acetyl groups or acetate. It is often presumed that most acetyl groups are on the xylan backbone, but some have also been reported to be acetylated for some plant species of lignin [173,174]. Acetyl removal from the xylan backbone was shown to enhance xylan hydrolysis due to increased xylan access, but not to directly impact glucan conversion [175]. Kumar and Wyman showed that selective deacetylation by the method developed by Kong *et al.* [176] enhanced cellbiohydrolase-I (CBHI) adsorption, increased the initial rate, and produced greater digestibility of cellulose and xylan, indicating increased cellulose accessibility and/or enzymes effectiveness [17,94]. This result led to the hypothesis that acetyl groups may restrict cellulase accessibility to cellulose by inhibiting productive binding through increasing the diameter of cellulose and/or changing its hydrophobicity [177].

Most of the studies reported above looked at the impact of hemicellulose removal on accessibility/digestibility for either agricultural residues or hardwoods, both of which have highly substituted xylan as a major hemicellulose component. Fortunately, the high dosages of cellulase and other commercial enzyme preparations often used, such as Novozyme 188, have a broad enough range of activities to hydrolyze carbohydrate polymers such as these completely [178]. However, hemicellulose in softwood, dried distiller's grains with solubles (DDGS), and corn fiber contains a wider range of components including (galacto)glucomannan, arabinogalactan, and arabinoxylan [179]. A multiplicity of enzymes is therefore required to realize high sugar yields [180], particularly at low enzyme loadings. In line with this reasoning, complexities in hemicellulose composition could be additional factors beyond the high lignin content often cited that result in higher enzyme loadings and much lower glucose yields from cellulose for softwoods compared to hardwoods or agricultural residues [181].

Role in Effectiveness

Removing hemicellulose not only enhances cellulose accessibility but greatly improves enzyme effectiveness. We recently showed for the first time that removing hemicellulose during pretreatment can reduce cellulase/xylanase inhibition by soluble xylooligomers generated during enzymatic hydrolysis [50,94]. Extension of these studies by Qing *et al*. and Qing and Wyman showed that xylooligomers inhibit cellulase even more than the well-established powerful inhibitor cellobiose at equal concentrations [51,52]. Previously, xylooligomers were demonstrated to inhibit endoxylanase action [182]. Although the effect of xylan removal on cellulase efficiency is still not completely established, it can be hypothesized to interfere with the processive action of Cel7A and reduce enzyme availability by binding cellulase unproductively [183,184].

During pretreatments, and especially batch pretreatments, hemicellulose degrades to furfural that further (and possibly hemicellulose directly) degrades to carbon-rich compounds called humins or pseudo-lignin [103,104,185]. For example, as shown in Figure 14.3, data adapted from Liu and Wyman [165] for batch hydrothermal pretreatment of corn stover at different temperatures showed that the amount of lignin removed with xylan solubilization increased and then decreased, possibly due to hemicellulose (xylan in this case) degradation to humins. The decrease in amount of lignin removed with xylan solubilized was sharp for pretreatment at high temperatures, possibly due to humins formation unless solubilized lignin precipitates more at high temperatures. Hemicellulose-derived humins, as recently discovered by Kumar *et al.* [103], can deposit onto (and/or co-exist with) cellulose and can affect both cellulose accessibility and enzymes effectiveness.

For enzymatic hydrolysis of lignocellulosics, deacetylation and removal of other side chains may indirectly affect cellulase effectiveness through removing bonds/linkages to xylose that xylanase could not otherwise hydrolyze, thereby making xylanase more effective [138,182,186–195] and in turn increasing cellulose digestibility [170,196,197]. Although the role of acetyl groups and other side chains is unclear, removal of such side chains during pretreatment would surely reduce enzyme requirements and enhance both xylan and glucan digestions as well [94,145,189]. Although not yet established experimentally, we

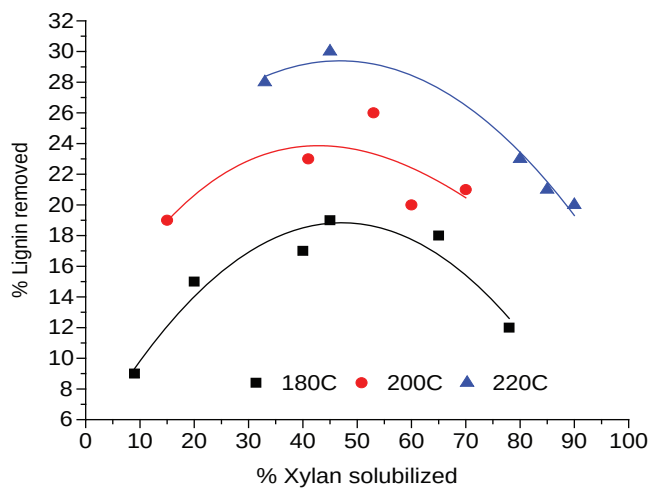

Figure 14.3 *Relationship between the amount of lignin removed (% of original) and xylan solubilized for batch hydrothermal pretreatment of corn stover performed at various temperatures and times (Data adapted from figure 14.6 in Liu and Wyman [165]).*

believe that removing arabinose substitution from the xylan backbone should impact accessibility and/or enzymes effectiveness due to its direct linkages with lignin.

14.4 Features Influencing Micro-accessibility and their Impact on Enzymes Effectiveness

14.4.1 Cellulose Crystallinity (Structure)

Role in Accessibility

Cellulose, which accounts for 25 to nearly 50% (w/w; dry basis) of lignocellulosic biomass, is composed of glucose molecules covalently bonded to one another in linear chains that are linked to other parallel cellulose chains in a highly ordered structure by hydrogen bonds [29,198–200]. However, glucose molecules in real cellulosic biomass are often collectively referred to as "glucan" because current compositional assays cannot accurately distinguish how much of the glucose entity is contained in cellulose versus hemicellulose (e.g., in xyloglucan, glucomannan). There are different allomorphs of cellulose (i.e., cellulose I, II, III [III_I and III_{II}], and IV [IV_I and IV_{II}]) that contain highly ordered crystalline and less-ordered amorphous regions [201–203]. Cellulose I, that has two polymorphs Iα and Iβ [204], is found in most of the plant cell wall with the amounts and type varying with anatomy (e.g., stalks, leaves, nodes, and internodes) and position in the plant cell wall [57,201,205]. For amorphous cellulose, the total number of hydrogen bonds per repeat units is 5.3 as compared to 9 in crystalline cellulose (Iα). Also, the cohesive energy density in the crystalline form is much higher than for amorphous cellulose, suggesting that non-crystalline forms of cellulose should be more reactive [206–208]. Although various methods (e.g., x-ray diffraction or XRD, nuclear magnetic resonance or NMR, Fourier transform infrared or FTIR spectroscopy [205,209]) have been applied to estimate cellulose crystallinity that indirectly indicates the extent of hydrogen bonding, most methods estimate cellulose bulk average crystallinity and do not distinguish differences in crystallinity (or the extent of hydrogen bonding) between/among different cell walls which may adversely affect cellulase accessibility/digestibility [40]. As an example, in their study Kataoka and Kondo showed that cellulose in the secondary cell wall is more crystalline than in primary walls [210,211].

Cellulase adsorption onto cellulose and cellulose moisture uptake are often reported to increase with decreasing crystallinity, indicating enhanced cellulose accessibility [16,212–214]. For example, Joeh *et al.* [40] showed that crystallinity greatly reduced adsorption of cellobiohydrolase I (Cel7A; CBHI), leading to a decreased extent of hydrolysis. In another study, although increasing crystallinity was reported to reduce the adsorption capacity of cellulose for complete cellulase [215], the maximum adsorption of purified exo- and endocellulases of *Irpex lecteus* protein could be inversely related to cellulose crystallinity [216,217], and more cellulose binding domains from cellulase adsorbed on amorphous than crystalline cellulose [218]. Working with a single source of cellulose, Hall *et al.* found that the cellulase adsorption increased as crystallinity decreased to an extent and then remained constant. Furthermore, the crystallinity value at adsorption saturation was a function of cellulase loading [219]. Nevertheless, contrary to the many other studies, when working with pure cellulose and lignocellulosic substrates Goel and Ramachandran did not find any correlation between crystallinity and cellulase adsorption as measured by enzyme activities in solution [220]. Interestingly, Banka *et al.* showed that adsorption of a non-hydrolytic protein increased with crystallinity [221].

Overall, the greater cellulase adsorption capacity of amorphous cellulose would lead one to expect amorphous regions to have greater hydrolysis rates and yields (by a factor of 2–25) than for crystalline areas [29,39,40,218,222–229]. The ordered structure of crystalline cellulose can therefore impact the ability of cellulase to access cellulose based on the mechanism that a layer of cellulose must be removed before enzymes can attack layers [208,230–232] and active sites lying underneath [223,233–235]. The observed slowdown in rates with increasing cellulose crystallinity, such as for the

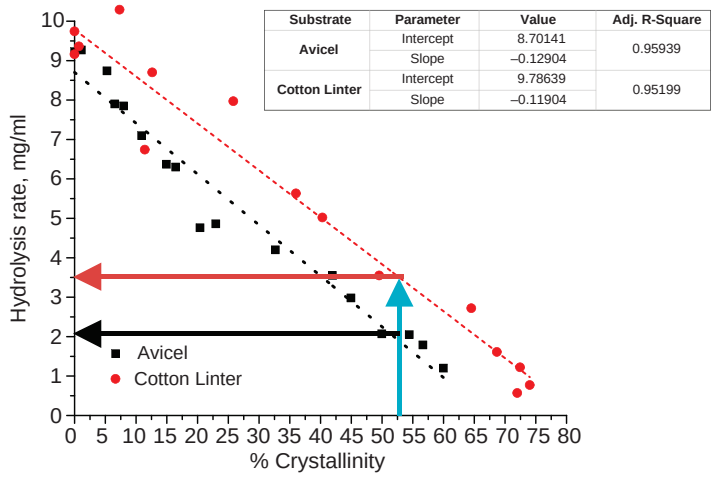

Figure 14.4 *Hydrolysis rate versus crystallinity for two cellulose substrates with different initial DPs. Cellulose samples of different crystallinities were prepared at varying phosphoric acid concentrations (Data re-plotted from Bansal* et al. *[236])*

example in Figure 14.4 for untreated and phosphoric-acid-treated Avicel and cotton linter [236], is consistent with this hypothesis [219,226,237,238].

However, others have observed the opposite effect for real biomass: hydrolysis performance improves with crystallinity [162,239,240]. Unfortunately, results with real biomass may be misinterpreted because removal of amorphous lignin and/or hemicellulose can increase biomass crystallinity and enhance digestibility. In some cases, cellulose crystallinity was considered to have no effect on hydrolysis rates [27,239,241–246]. One explanation could be that greater moisture uptake does not necessarily mean enhanced hydrolysis rates, as the cellulase molecular weight is about 3000 times that of water.

If hydrolysis rates are much slower for crystalline regions and amorphous regions are preferentially hydrolyzed, the classical question arises as to why crystallinity does not increase over the course of cellulose hydrolysis as a result of more rapid removal of amorphous cellulose [225,247]. However, no significant change in crystallinity has been measured over the course of cellulose hydrolysis [219,248–252]. Working with a bleached softwood Kraft pulp (albeit at a high enzyme loading of about 166 IU/g glucan), Pu *et al.* showed preferential action by cellulase on non-crystalline cellulose regions, cellulose Iα polymorph, and para-crystalline cellulose compared to cellulose Iβ polymorph and highly crystalline cellulose regions only during the initial phase of hydrolysis, during which cellulose crystallinity increased to some extent [253]. However, after the initial phase, all regions were similarly susceptible to enzymatic hydrolysis with little change in crystallinity. Consistent with this observation, Hall *et al.*, Cateto *et al.* and Penttila *et al.* recently showed similar findings in their respective studies [219,252,254]. The following points may help understand this conundrum and possible mechanisms [16].

1. High enzyme loadings, as often employed to determine the impact of biomass features and other factors on hydrolysis, may lead to misinterpretation by saturating the substrate and impacting other biomass features with enzymes activities other than cellulase in commercial preparations. In particular, fungal cellulases have >50% of their total protein designed to attack and deconstruct crystalline cellulose, with the result that high enzyme loadings would make it hard to differentiate the effect of crystallinity (or modest changes in crystallinity, as often misinterpreted) on hydrolysis.

2. As demonstrated in recent studies, cellulolytic components not only function as hydrolytic agents but can simultaneously disrupt cellulose structure to a significant extent [255–261]. Thus, during hydrolysis, the action(s) of individual monocomponent enzymes is likely obfuscated by concurrent modification by complementing enzymes [255]. Park *et al.* addressed this phenomenon by stating that: "*if the enzymes work ablatively on cellulose microfibril surfaces, consuming the less ordered surface layers of cellulose, then internal ordered cellulose chains will become surface chains with decreased order, so that conversion of amorphous cellulose results in production of more 'amorphous cellulose' and a further decrease in cellulose CI*" [205].
3. For real biomass, crystallinity should not be confused with absolute cellulose crystallinity as real biomass has amorphous components other than cellulose [28,209,242].
4. Almost all the characterization methods require treatment before analysis such as drying or coating that may disturb the structure of biomass [29,209]. Also different analytical methods give different crystallinity values; hence the outcome must be interpreted with caution [29,199,205,209,262].

Nonetheless, a better understanding of cellulase behavior at the micro level and advanced analytical tools would help to understand the role of crystallinity/structure in controlling cellulose micro-accessibility.

Role in Enzymes Effectiveness

From the previous section, it appears that cellulose crystallinity plays a very important role in cellulose accessibility; however, cellulose crystallinity would likely impact the effectiveness of adsorbed cellulase components as well. The latter became more evident from a first-of-its-kind of study by Hall *et al*. in which they showed that cellulase adsorption increased with a reduction in crystallinity only up to a point. However, the hydrolysis rate kept increasing with further reductions in crystallinity while adsorption remained constant [219]. This study directly supports the hypothesis we stated earlier that crystallinity impacts both accessibility and effectiveness [16,263]. Furthermore, the literature showed that cellulose crystallinity affects synergism between cellulase components [29,219,232,264–271]. Because crystalline cellulose is highly hydrophobic, it can irreversibly bind cellulase; this lowers enzyme effectiveness as surface-bound CBHI may lose up to 70% of its activity in only 10 minutes [272]. Crystallinity can therefore impact enzyme effectiveness.

Other than cellulose bulk crystallinity, the cellulose types and crystalline cellulose polymorphs may also affect enzyme effectiveness. Igarashi *et al*. showed that the maximum cellulase adsorption capacity on cellulose Iβ was approximately 1.5 times that for cellulose Iα, although the rate of cellobiose generation from cellulose Iβ was lower than that from cellulose Iα [273,274]. In another study, Igrashi *et al.* showed that the activation of cellulose I to cellulose III_I by ammonia treatment resulted in 5 times more sugar generation, with twice the amount of cellulase adsorbed [275]. Consistent with this, by employing molecular dynamics simulations Chundawat *et al.* recently showed that ammonia treatment converts cellulose I to cellulose III by redistributing the hydrogen bonds to enhance cellulose accessibility and enzyme effectiveness [276].

14.4.2 Cellulose Chain Length/Reducing Ends

Role in Accessibility

Information on the effect of cellulose degree of polymerization (DP) on cellulose accessibility is very limited. However, based on catalytic preferences of cellulase exoglucanases [277–281], it appears that the greater the number of reducing (non-reducing) ends, the greater should be cellulose accessibility. The ambiguity surrounding the effect of cellulose chain length on accessibility to enzymes is partly due to its close

association with cellulose crystallinity and particle size. For example, mechanical pretreatments such as ball milling generally reduce particle size, crystallinity, and DP [215,226,228,282,283] for pure cellulose and could also affect lignin structure in real biomass. On the other hand, thermochemical pretreatments (especially at low pH) by such options as steam explosion, dilute acid, and other chemical/physical treatments such as commonly used acid-chlorite delignification [54,55], significantly affect cellulose DP in addition to several other features, again making it difficult to differentiate control [7,28,284–286]. Due to the complexity of real biomass and close association with other physiochemical characteristics, it is therefore difficult to differentiate the impact of cellulose DP on accessibility.

Although conclusive studies directly showing the impact of cellulose chain length on accessibility are scarce, Kaplan *et al.* [287] found a significant drop in cellulase adsorption and hydrolysis of altered cellulose following photochemical degradation, probably due to a decrease in cellulose DP and some ring opening for weathered cotton cellulose. Although cellulase adsorption was not measured to determine its impact, a few other studies indirectly showed the impact of cellulose chain length on cellulose accessibility. For instance, working with wheat straw and bagasse Puri and Pearce [239,245] showed that a reduction in cellulose DP improved hydrolysis, but the impact of features other than crystallinity was not studied. Using highly crystalline bacterial cellulose (BC), Väljamäe *et al.* showed the impact of cellulose DP on both CBHI (Cel7A) and endoglucanase I (EGI) (Cel7B) activity [232]. Treating highly crystalline BC with 1 M hydrochloric acid resulted in little change in cellulose crystallinity, but a major reduction in DP (from 2620 to 150 in 40 min). The reduction in DP increased CBHI relative activity from 37% to 100% but reduced EGI relative activity from 100% to 50%. However, longer incubation of BC with HCl dropped the DP from 150 to 114, with a somewhat negative impact on CBHI and a positive effect on EGI relative activities [232]. For subcritical water pretreatment of Avicel cellulose, Kumar *et al.* reported that microcrystalline cellulose DP decreased dramatically from *c.* 320 to *c.* 100 at temperatures above 300 °C, along with a slight transformation of cellulose I to cellulose II but no change in crystallinity, apparently resulting in increased hydrolysis rates and yields [288]. Hu *et al.* and Hu and Ragauskas and showed that the enzymatic digestibility of cellulose in leaves was much higher than in internodes for hydrothermal pretreatment despite similar cellulose and lignin structures and profiles except cellulose DP, which was much lower for cellulose in leaves [285,289]. Cellulose decrystallization via ball milling is often accompanied by particle size and cellulose chain length reduction, making it difficult to isolate the effect of either feature. However, Bansal *et al.* applied different phosphoric acid concentrations to alter the crystallinity of two different cellulose types: Avicel (CrI *c.* 60%) and cotton linter (CrI *c.* 72%). Their data, re-plotted in Figure 14.4, implied that cotton linter with lower DP of about 180 [217] had a higher cellulose hydrolysis rate than Avicel (DP *c.* 350) at any given crystallinity, assuming that phosphoric acid treatment had no or similar relative effects on chain length for both cellulose types. From correlations in Figure 14.4 it appears that at 0% crystallinity, the enzymatic hydrolysis rate for cotton linter and Avicel would be 9.78 mg/mL and 8.70 mg/mL, respectively.

Kasprzyk *et al.* showed that gamma radiation below 120 kGy did not have a significant impact on cellulose morphology and crystallinity [290]. Consistent with this, Katsumata *et al.* applied gamma radiation of 100 kGy to sapwood and showed enhanced termite feeding activity, mainly due to decreased cellulose DP resulting from gamma radiation [291]. In another study, Knappert and coworkers developed a qualitative relationship between cellulose DP and digestibility [292]. However, Sinistyn *et al.* contradicted these conclusions by showing that a reduction in DP of cotton linters by application of gamma irradiation while keeping crystallinity index (CI) constant had a negligible impact on hydrolysis rates [226]. Furthermore, a kinetic study by Zhang and Lynd indicated that reducing cellulose DP had less effect on accelerating hydrolysis rates than increasing accessibility of β-glycosidic bonds as measured by the maximum amount of cellulase adsorbed on cellulose [293]. However, the possibility of cellulose

chain length (DP) impacting accessibility was not discussed. On a different note, cellulose surface properties (free energy [$gS = 37.56 + 0.02$ DP] and polarity [$P = 11.88–0.02$ DP $+ 9.10$ DP^2] were related to cellulose chain length [294] and both were correlated to increase with cellulose DP [294].

Role in Effectiveness

Other than impacting accessibility, cellulose DP can also affect enzyme effectiveness; relevant literature is once more limited, however. Hypothetically, the lower the DP, the more reducing and non-reducing ends are available, and more CBHI/II would be expected to be able to work at one time while exposing more sites for endoglucanases attack. However, as cellulose deconstruction is assumed to be a surface phenomenon and the mechanism is often characterized in terms of a peeling action [252,295], the surface availability of reducing/non-reducing ends can be limited [234,235] and affected by other structural features, as discussed above. Nidetzky *et al.* found that, for soluble cellulose, the initial degradation velocity of cello-oligosaccharides by CBHI increased with DP below cellohexose but was constant for higher DP [281]. Similar DP effects of soluble cellodextrins on CBHII and EGI activity have been reviewed elsewhere [29]. Furthermore, a decrease in β-glucosidase activity with increasing DP has been reported [296,297]. To the authors' knowledge, not much information is available on the effect of insoluble cellulose DP on the catalytic efficiency of cellulose, except that higher DP could result in higher synergy between CBHI and EGI [268,293,298,299]. However, Gupta and Lee showed that cellulase could hydrolyze non-crystalline cellulose (NCC) with high yields when cellulose DP was large, because the CBHI component of the cellulase system cannot recognize substrates with a DP below 10; this led to the result that cello-oligosaccharides with DP < 10 and > 3 are not hydrolyzed [35]. Furthermore, cellulose DP may affect the processivity index, with full processivity of CBHI possibly not realized for short chains [35,232]. Overall, studies of the effect of DP and crystallinity on enzymatic digestibility demonstrated that the susceptibility of pretreated substrates to enzymatic hydrolysis could not be easily predicted from differences in their cellulose DP and crystallinity [239,300], likely due to the complexity of real cellulosic substrates.

14.5 Concluding Remarks

In this review chapter based on the information in literature and findings in our lab on strong correlations between maximum cellulase (complete or a component) adsorption capacity and hydrolysis rates/yields [17,106,301,302], such as for the example in Figure 14.5, it was hypothesized that enzymatic hydrolysis of cellulose in pretreated biomass is controlled by two main factors: enzyme accessibility to cellulose and enzyme effectiveness on cellulose. However, accessibility can further be visualized in two categories: macro-accessibility and micro-accessibility. The factors influencing macro- (mainly lignin and hemicellulose) and micro-accessibility (cellulose crystallinity and type and chain length) most and their impacts on enzymes effectiveness discussed in this chapter are summarized in Table 14.1. Due to complexity of actual cellulosic biomass materials, it is difficult to assign some features to a single category as they can likely affect both macro- and micro-accessibility.

Some other biomass features that are also believed to affect enzymatic hydrolysis such as biomass porosity and pore volume, biomass and cellulose surface area, and particle size [72,161,303] were not included due to their lower impact on hydrolysis for real biomass, their non-exclusive behavior, or inadequate evidence to support our hypothesis. For example, several studies showed that biomass porosity/pore volume, which should not be confused with micro/nanopores or cracks within cellulose microfibrils [142,304,305], play a major role in accessibility and enzymes effectiveness due to the molecular weights/sizes of cellulolytic components. Although pores/porosity appear to play a vital role, unpretreated cell walls have a porous structure to support plant growth that no doubt changes during pretreatment, and an increase in porosity is

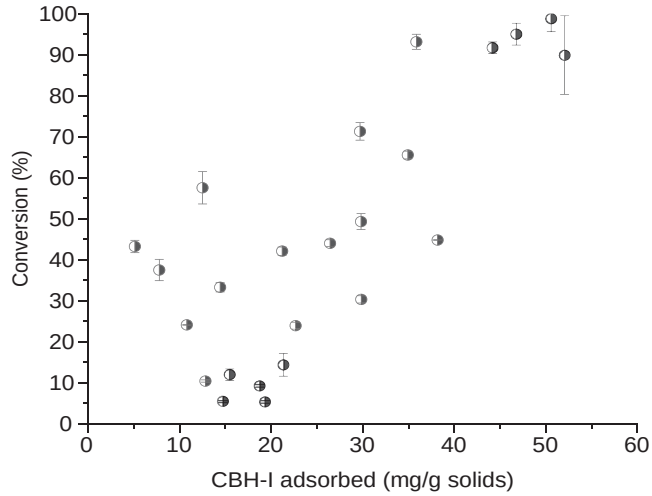

Figure 14.5 *The 24-h cellulose conversion (%) versus CBH-I adsorption (mg/g solids) for Avicel cellulose and poplar solids prepared by various leading pretreatments and those solids hydrolyzed to different extents (Data adapted from Kumar and Wyman [42]).*

often concomitant with changes in other biomass structural features such as hemicellulose and pectin removal during pretreatment [150,306,307]. It is therefore difficult to ascertain the influence of porosity on accessibility and enzyme effectiveness. Other than that, the methods employed to measure porosity and pore volumes do not appear to be reliable enough. However, although difficult to measure for real biomass, cracks and micro/nanopores may also exist within cellulose microfibrils but, according to the current picture of cellulase taking cellulose apart layer by layer, cracks and nanopores within microfibrils may have a limited effect on accessibility but could impact effectiveness. For example, Tanka *et al.* showed that cellulase components trapped within pores may have low synergism and thus lower hydrolysis yields/rates [305]. On the contrary, working with several types of pure cellulose Gama *et al.* showed that cellulase does not enter these micropores [304]. Consistent with this, Penttila *et al.* recently reached the same conclusion, namely: "*enzymes act on the surface of cellulose bundles and are unable to penetrate into the nanopores of wet cellulose*" [254].

Table 14.1 *Summary of pretreated biomass key chemical and physical features and their role in cellulose macro-/micro-accessibility and enzyme effectiveness.*

Biomass chemical/physical features	Role in cellulose accessibility		Role in enzyme effectiveness
	Macro	Micro	
Lignin	Major (apparently indirect[a])	Minor, if any	Significant
Hemicellulose	Major (direct)	Minor, if any	Major
Acetyl/arabinan/other substitution on xylan backbone	Significant[a]	Minor, if any	Significant
Crystallinity	Minor, if any	Major	Major
Cellulose chain length/ DP	Minor, if any	Possibly significant[a]	Possibly significant

[a] Hypothesis needs more research.

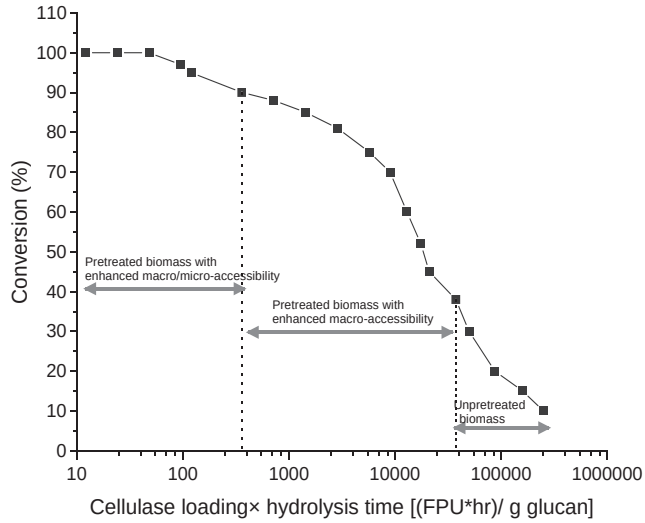

Figure 14.6 *Hypothetical depiction of cellulose conversion versus cellulase loading × hydrolysis time (FPU hr/g glucan) with changes in macro-/micro-accessibility in pretreated biomass.*

Biomass particle size is often suggested to affect enzymatic hydrolysis by increasing the surface area for greater enzyme adsorption, based on data with pure celluloses [220,308–310]. However, particle size does not appear to play a plausible role for real biomass unless one of the other major features (either lignin or hemicellulose) impacting cellulose macro-accessibility is altered [311]. In addition, data on the effect of particle size should be interpreted cautiously as mechanical size reduction may also affect other structural features.

Understanding the effect of surface area, often deemed responsible for effective enzymatic hydrolysis, is again marred by the measurement techniques applied and interpretation of the data. For example, Gupta and Lee showed that BET surface area of NCC almost doubled compared to the original untreated cellulose; however, hydrolysis rates for NCC were almost 10 times higher [35]. Together, we believe that the features described here contribute directly or indirectly to cellulose accessibility and enzyme effectiveness, but the extent of each is still debatable.

The key question, however, is which accessibility categories or biomass features and their alteration affect cellulose hydrolysis the most. The choice will depend strongly on biomass type and the desired outcome. For instance, impacts on either accessibility category can achieve reasonable sugar yields with a somewhat high enzyme loading; however, they must both be changed to realize acceptably high sugar yields with very low enzyme loadings and short processing times, as illustrated in Figure 14.6.

Most leading pretreatments, except for those at low pH, seem to affect features in the macro-accessibility category and apparently enhance biomass digestibility by changing mostly cellulose macro-accessibility through altering/removing either hemicellulose or lignin or both. Although solids pretreated by these techniques are highly digestible, high and yet economically unfeasible enzyme loadings are required to realize these high yields. Conversely, other pretreatments such as concentrated phosphoric acid (COSLIF) [312,313] and ionic liquids [314–316] appear to enhance both macro- and micro-accessibility and thus require less enzyme and processing time compared to pretreatments that impact only macro-accessibility However, changing only micro-accessibility (such as through ball milling to alter cellulose

structure/crystallinity) does not lead to effective conversions at low enzyme loadings (personal communication with Dr Mark Holtzapple, Texas A&M, 2011). In addition, COSLIF/ionic-liquid pretreatments that strongly address micro-accessibility need to remove/relocate hemicellulose/lignin (i.e., address macro-accessibility) to realize higher sugar yields with low FPU hr/g glucan [199,209,317,318]. Furthermore, even within the macro-accessibility category, the effectiveness of removing lignin or hemicellulose depends on biomass types.

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