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Substrate-Related Factors Affecting Enzymatic Saccharification of Lignocelluloses: Our Recent Understanding

Shao-Yuan Leu • J. Y. Zhu

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Abstract Enzymatic saccharification of cellulose is a key step in conversion of plant biomass to advanced biofuel and chemicals. Many substrate-related factors affect saccharification. Rather than examining the role of each individual factor on overall saccharification efficiency, this study examined how each factor affects the three basic processes of a heterogeneous biochemistry reaction: (1) substrate accessibility to cellulose—the roles of component removal and size reduction by pretreatments, (2) substrate and cellulase reactivity limited by component inhibition, and (3) reaction conditions-substrate-specific optimization. Our in-depth analysis of published literature work, especially those published in the last 5 years, explained and reconciled some of the conflicting results in literature, especially the relative importance of hemicellulose vs. lignin removal and substrate size reduction on enzymatic saccharification of lignocelluloses. We concluded that hemicellulose removal is more important than lignin removal for creating cellulase accessible pores. Lignin removal is important when alkaline-based pretreatment is used with limited hemicellulose removal. Partial delignification is needed to achieve satisfactory saccharification of lignocelluloses with high lignin content, such as softwood species. Rather than using passive approaches, such as washing and additives, controlling pretreatment or hydrolysis conditions, such as pH, to modify lignin surface properties can be more efficient for reducing or eliminating lignin inhibition to cellulase, leading to improved lignocellulose Saccharification.

Keywords Enzymatic hydrolysis/saccharification · Pretreatment · Biofuel and biorefinery · Lignocelluloses Cellulase enzymes · Lignin · Accessibility

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S.-Y. Leu · J. Y. Zhu
USDA Forest Service, Forest Products Laboratory,
Madison, WI, USA

J. Y. Zhu (✉)
Dept. of Biological Systems Engineering,
University of Wisconsin-Madison,
Madison, WI, USA
e-mail: jzhu@fs.fed.us

J. Y. Zhu
e-mail: jzhu9@wisc.edu

Introduction

Enzymatic saccharification of cellulose is one of the key, as well as costly, steps for biofuel production from lignocellulosic biomass [1]. High cellulase dosing, on the order of 10–25 g protein (or 10–25 kFPU)/kg of biomass, is required to achieve satisfactory saccharification using current and promising technologies [2]. Fundamental understandings of enzymatic saccharification of lignocelluloses are critical to reducing cellulase loading and improving sugar/biofuel production from lignocelluloses. Many studies have identified the critical parameters that affect enzymatic saccharification of lignocelluloses. These parameters were classified into two basic subjects, i.e., the substrate-related and enzyme-related parameters [3, 4]. The subject of enzyme-related parameters has been extensively studied, and a good understanding has been obtained [5–7]. The subject of substrate-related parameters has also been extensively studied [8, 9]. For pure cellulosic substrates, the major substrate structure features are: (1) the capillary (or pore) structure of cellulosic fibers [10, 11], (2) crystallinity [4, 10], and (3) particle size or external specific surface area [12–14].

Lignocellulosic biomass (i.e., corn stover, switch grasses, and wood or forest residues) has much more complex physical and chemical compositions than pure cellulosic fibers, and is more difficult to hydrolyze; the structure of lignocellulosic biomass is more compact than pure cellulosic fibers, and the cellulose is better protected by the cell wall.

Enzymatic saccharification of lignocelluloses substrates is affected by many substrate-related physical and chemical parameters, such as native (untreated) wood lignin content, the extent of lignin and xylan removal by pretreatment [15–19], lignin structure [16, 20–22], substrate size [12, 13, 23–26], and of course substrate pore surface area or substrate accessibility to cellulase.

Published studies of the individual substrate parameters on enzymatic saccharification of physical and/or thermochemical pretreated lignocelluloses do not always present consistent conclusions [27]. For example, some studies showed that substrate enzymatic digestibility (SED), defined as the percentage of substrate cellulose enzymatically saccharified, is directly correlated to residual lignin content in the pretreated biomass [17, 28–31]. For hardwood species, hemicellulose removal or reduction in xylan content was found to be proportional to saccharification, and both residual lignin content and lignin removal were not important [18, 19]. Other studies, however, showed that the cellulose accessibility to cellulase (CAC) had greater influences on enzymatic hydrolysis than lignin removal [32–34]. Inconsistent results were also presented on the effect of biomass size reduction on enzymatic saccharification of lignocelluloses [13, 24, 26, 35]

Enzymatic hydrolysis of lignocelluloses is a heterogeneous biochemical reaction. Like any heterogeneous reaction, the three important processes are (1) the intimate contact of the reactants, i.e., cellulose accessibility and binding of cellulase to cellulose [36], (2) the reactivity of the reactants, e.g., cellulase activity and cellulose crystallinity, and (3) the reaction conditions, such as temperature, pH, etc. Most substrate-related parameters are associated with (1) the accessibility of cellulose to cellulase. The enzyme-related parameters are mostly associated with (2) the cellulase reactivity. Rather than examining the role of each individual factor on overall saccharification efficiency, this review uses a holistic approach to examine how various substrate-related parameters affect the three basic processes, especially process (1), of the heterogeneous reaction of enzymatic hydrolysis based on recent literature data. The objective is to unify or reconcile some of the conflicting results in the literature on enzymatic saccharification of lignocelluloses and to obtain an improved understanding of various pretreatment processes and their effectiveness for removing lignocellulose recalcitrance.

Contact of Cellulase with Cellulose–Cellulose Accessibility to Cellulase

Intimate contact between enzymes and cellulose is the most critical process affecting heterogeneous biochemical reaction between them [27, 34, 36]. This is especially true for enzymatic saccharification of lignocelluloses because cellulose accessibility is further limited by the non-cellulosic components, such as hemicelluloses and lignin. In this section, we will first review the determination of substrate accessibility or CAC. We will then examine several recent studies on the effects of substrate accessibility on cellulose saccharification efficiency. In contrast to most published works [15-20], these recent studies used well-defined substrates with substrate accessibility or CAC being the only variant, helping to illustrate the direct cause–effect relation between CAC and SED. Using primarily recent literature, we will also examine this relationship for substrates produced by different pretreatment processes under varying conditions. This will relate individual lignocellulose component removal and size reduction to substrate accessibility or CAC.

Measurement of Substrate and Cellulose Accessibility

The term substrate accessibility should be differentiated from the meaning of CAC. Whereas CAC is limited to the cellulose surface accessible to cellulase, substrate accessibility also includes non-cellulosic surfaces and is dependent on the probe molecule used. The accessibility of a substrate can be quantified by the accessible specific total pore volumes (cubic meters per gram), or specific surface areas (square meters per gram), for a given probe molecule used. Only when the given probe molecule is a cellulase, or is a representation of a cellulase, can the measured cellulose accessibility be defined as CAC––cellulose accessibility to cellulase.

There are two general approaches to evaluate the lignocellulosic substrate accessibility. The first approach directly measures the accessible volume (porosity) or surface area of a substrate using one or a set of probing molecules. The probe molecule can be water as in the Water Retention Value (WRV) method [37, 38], differential scanning calorimetry, and NMR porosimetry [39-41], or a set of dextran molecules as in the classical solute exclusion technique [42, 43]. The second approach measures the adsorption of a given molecule to a lignocellulosic substrate as in the classic BET method which measures the adsorption of nitrogen by the pore surfaces [44, 45], the Simons' staining method which measures the adsorption of dyes [46, 47], and protein or cellulase adsorption methods that directly measure the amount of proteins or cellulase adsorbed onto a lignocellulosic substrate [32, 37, 48–50]. To determine CAC, the non-cellulosic surface needs to be blocked and excluded from measurements. Furthermore, a probing molecule needs to have a cellulose binding module (CBM) to simulate cellulase binding to cellulose and must have a molecular size similar to that of cellulase at approximately 51 Å [36]. These requirements suggest that the adsorption methods using either a thioredoxin–green fluorescent protein–CBM3 (TGC) protein with a CBM [48, 50] or directly using a

cellulase [49, 51] are the two viable methods for accurately determining CAC. Both of these methods use bovine serum albumin to block non-cellulosic surfaces [52].

Substrate or Cellulose Accessibility and Enzymatic Cellulose Saccharification

Two recent studies were conducted in our laboratory using several sets of substrates, each set derived from a never-dried or a never-pressed pretreated lignocellulosic sample by drying or wet pressing to varying solid levels [38, 53]. The chemical structure of a given set substrate was identical, and the only difference was the pore sue or surface area. This is because either drying or pressing only causes the collapse of pores—fiber hornification due to hydrogen bonding with minimal change of external surface [54]. Therefore, the direct cause–effect relation between substrate/cellulose accessibility and saccharification can be observed for these substrates. It was found that for a given set of substrates produced from the same never-dried or never pressed sample, cellulose enzymatic saccharification efficiency, reported as SED, is directly proportional to the pore volume of the substrate measured by WRV, and independent of the drying temperature or duration [38]. WRV represents the total substrate surface area accessible to water molecules.

A follow-up study was also conducted in our laboratory recently which more quantitatively evaluated the relation between SED and substrate accessibility or CAC [49]. The substrate accessibility and CAC of the set of substrates, produced from Sulfite Pretreatment to Overcome Recalcitrance of Lignocelluloses (SPORL) pretreatment of never-dried lodgepole pine [55], were measured by the solute exclusion [43] and TGC protein technique [48, 50], respectively. The SED increased linearly with the increase in the accessible pore surface measured by solute exclusion to a 51 Å molecule (Fig. 1). Furthermore, the study also found a greater than 90 % reduction in SED when the substrate is completely hornified, i.e., all pores were collapsed with a degree of hornification=1. This is in agreement with an early study by Wong et al. [56]. This suggests that substrate pore (internal) surface area contributed to more than 90 % of the cellulose saccharification and that the substrate external surface played a minor role for this given SPORL. pretreated sample. Similar relationships were also obtained between SEDs and the adsorption amounts of a commercial endoglucanase for two sets of substrates produced by wet-pressing of a pretreated lignocellulosic and bleached pulp samples [53]. The linear relation between SED and substrate accessibility clearly indicates that accessibility is the dominant factor affecting cellulose saccharification of lignocelluloses. Furthermore, there is no diminishing effect on enzymatic cellulose saccharification by improving substrate CAC.

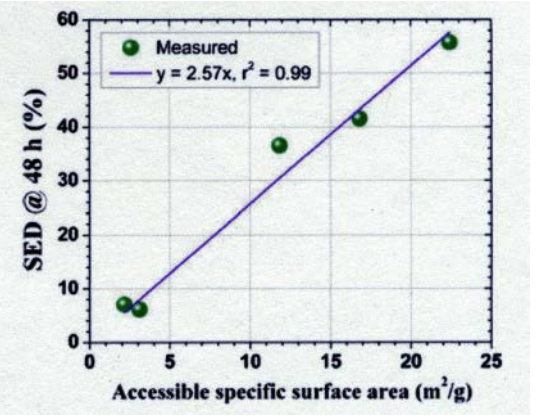

Fig. 1 Linear relation between solute exclusion measured substrate accessibility using molecular size 51 Å and substrate enzymatic digestibility (SED) for a set of substrates produced by drying a never-dried SPORL pretreated lodgepole pine substrate to different degrees. Substrates from [38] and data from [49]

Thermochemical Pretreatment and Removal of Individual Cell Wall Component to Improve Substrate/Cellulose Accessibility

One way to improve CAC is to deconstruct the cell wall of lignocellulosic biomass through thermochemical pretreatment. Pretreatment removes cell wall components which result in a substrate with a relatively open and porous structure. Thermochemical pretreatment also changes the chemical structure of lignocelluloses, which complicates the understanding of the role of CAC on cellulose saccharification. Direct cause-effect relation between CAC and SED, as discussed in the previous subsection, cannot be easily obtained for substrates produced using different pretreatments or under different pretreatment conditions using the same pretreatment. However, good correlations between accessibility or CACs and SED have been reported in several early studies [56–60]. Furthermore, despite the high lignin content of a softwood species studied, SED was found independent of the amount of lignin removal by SO_2-catalyzed steam explosion and almost inversely proportional to the resultant substrate lignin content [56]. Similar results were also reported recently when different pretreatment processes were applied to lodgepole pine [49]. These observations agree with a recent study [34] and further corroborate that the primary role of pretreatment is to increase substrate accessibility or CAC for effective lignocellulose saccharification.

Elucidating the effect of removal of individual component by pretreatment can help to quantify the importance of substrate accessibility and the effectiveness of different pretreatments for improving enzymatic saccharification of lignocelluloses. Current understanding of cell wall structure is limited [61]. However, it has been generally recognized that the dominant structure is represented by elemental

cellulose fibrils being cross-linked by hemicelluloses and embedded in a non-cellulosic polysaccharide matrix of hemicelluloses and pectin to form microfibrils [61–65]. Lignin, as a plasticizer, is part of the non-cellulosic matrix but not directly cross-linked with cellulose chains on the surface of the elemental fibrils. Based on this understanding, it is clear that the removal of hemicelluloses is more important to improving cellulose accessibility than the removal of lignin. We will illustrate this point using results from recent studies using two general types of lignocellulosic feedstocks having either a low or high lignin content.

For most low lignin content lignocelluloses, such as corn stover and switchgrass, significant removal of hemicelluloses is sufficient to achieve excellent enzymatic saccharification of celluloses without delignification (only solubilizing acid-soluble lignin) [20, 66,67]. In a recent study, we found cellulose saccharification efficiency (SED) of aspen (wood lignin content of only 20.8 %), pretreated by dilute acid and SPORL under different conditions, was linearly proportional to the amount of xylan removal (Fig. 2a) [18]. However, the same SED data for the 51 substrates were independent of lignin removal as shown in Fig. 2b. As a matter of fact, the data in Fig. 2b suggest that cellulose saccharification has a general trend of an inverse relationship with lignin removal (there are no data points in the lower left quarter of the plot). This is because increased lignin removal was achieved at the expense of hemicellulose removal for acid-based pretreatments due to lignin condensation (pH of all pretreatments were below 2.5). In the case of SPORL high pH pretreatments (conducted at pH of approximately 4), there appears to be no correlation between SED and lignin removal (Fig. 2b). Only when comparing several substrates with approximately the same amount of hemicellulose removal of 85 % can the effect of increased lignin removal for improving cellulose saccharification be observed (Fig. 2c). This implies that lignin removal plays a secondary role. A similar study using eucalyptus produced very similar results [68], i.e., the SEDs of 47 eucalyptus substrates produced by dilute acid and SPORL pretreatments under different conditions were proportional to xylan removal and independent of lignin removal.

We can also support the argument that delignification is less important than hemicellulose removal using data from a set of pretreatments on lodgepole pine (softwood with a high wood lignin content of 27.1 %) conducted under different conditions. SPORL pretreatments, using different pH and resulting in different levels of delignification and hemicellulose removal, along with a dilute acid pretreatment were employed. SED of these pretreated lodgepole pine substrates increased almost linearly with the hemicellulose (xylan+mannan) removal while having significant variations in lignin removal ranging from 1 to 44 %; however, this did not apply for the dilute acid sample and one case of

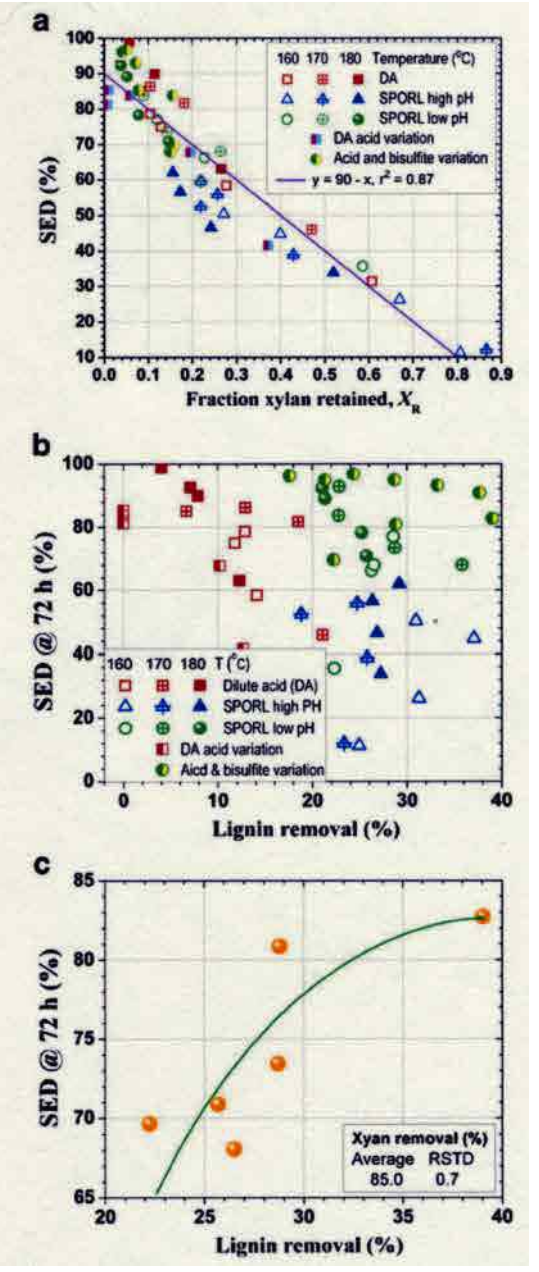

Fig. 2 Effects of xylan and lignin removal on substrate digestibility (SED) for pretreated aspen substrates. **a** Xylan removal effect for all 51 substrates produced by dilute acid and SPORL pretreatments under different conditions. Figure from [18]; **b** Lignin removal effect for the same 51 substrates in **(a). c** Lignin removal effect for six substrates having approximately the same xylan removal of 85 % with relative standard deviation (RSTD) of 0.7 %. Substrates and data from [18]

SPORL (pH= 1.8), both pretreated substrates with very low lignin removal at less than 16 % (Fig. 3, data from [69]). SED increased with the increase in lignin removal only up to 30 %. Further increases in lignin removal decreased SED due to the decrease in hemicellulose removal when a slightly higher pH was used. This suggests that a minimum amount

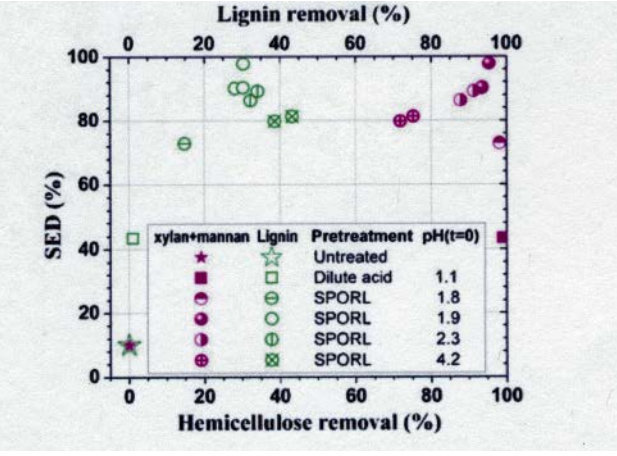

Fig. 3 Effects of lignin and hemicellulose removal by different pretreatments on substrate digestibility (SED) for pretreated lodgepole pine substrates. Substrates and data from [69]

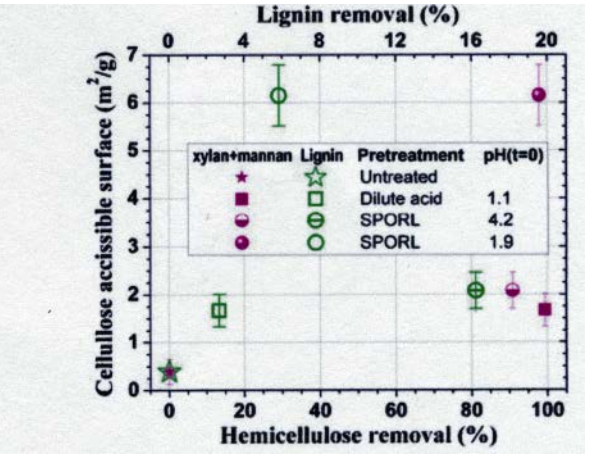

Fig. 4 Effects of lignin and hemicellulose removal by different pretreatments on cellulose accessible surface of pretreated lodgepole pine substrates. Substrates and data from [24]

of lignin removal (20 % for this case, or approximately 5.5 % of the total mass) is required for lignocelluloses with high lignin content. However, the dependence of lignocellulose saccharification efficiency on hemicellulose removal is apparent and independent of the lignin removal once this minimal level of delignification is achieved.

The results from softwood lodgepole pine, presented above, suggest that the relative importance of removing a specific biomass component to improve cellulose accessibility is partially dependent on the coverage of the component over cellulose fibrils in a given feedstock. The relative importance of delignification for enhancing enzymatic saccharification increases for softwood species when compared with hardwood species [17, 70]. However, complete delignification was not necessary to achieve significant enzymatic saccharification of softwood pulp with partial hemicellulose removal [17, 55, 69]. Substrate pore surface measurements indicated that removal of the same amount of lignin by different methods did not produce the same amount of pore surface. A recent study on alkaline pretreatment of softwood and hardwood indicated that delignification process that produced less pore surface resulted in lower cellulose saccharification than delignification that produced more surface area at the same level of lignin removal [17]. The relative importance of removing lignin versus hemicelluloses can also be seen by comparing the cellulose accessible area of lodgepole pine substrates produced by different pretreatments (Fig. 4) with data from [49] and [24]. When comparing the two SPORL pretreatments, i.e., SPORL@pH=1.9 and SPORL@pH=4.2, the additional 7 % hemicellulose removal by SPORL@pH=1.9 is more important, i.e., increased CAC by 200 %, than the additional 10 % lignin removal by SPORL@pH=4.2. When comparing dilute acid with SPORL@pH= 1.9, both having near complete hemicellulose removal of approximately 98 %, the approximately 4 % additional lignin removal by SPORL increased CAC by approximately 200 %.

Lignin coverage can have an effect on increasing substrate accessibility as illustrated by the relative importance of delignification for softwoods discussed above. This point is better illustrated using results from wood of the same family. When four poplar clones (native lignin content from 20.2 to 25.2 %) were pretreated under the same conditions using dilute acid and SPORL, the hemicellulose removal was inversely proportional to wood lignin content [19]. This is in agreement with a more comprehensive study using hundreds of poplar clones [16]. This suggests that native lignin plays a critical role in protecting carbohydrate from degradation. But this protection varies with lignin type. For example, guaiacyl lignin present in both hardwood and softwood species is more strongly cross-linked and, therefore, more resistant to chemical degradation [71].

Delignification is always more difficult and expensive than hydrolysis of hemicelluloses based on pulping research and practice, and is consistent with the role of lignin to protect against carbohydrate degradation. This suggests that delignification breaks down the strong structure of cell wall, while hydrolysis of hemicellulose breaks down the weak structure of cell wall. This is corroborated by the results presented above. It can be therefore concluded that delignification through alkaline-based pretreatments is less effective than hydrolysis of hemicelluloses using acid for increasing cellulose accessibility to cellulase. It also suggests that increasing substrate accessibility depends on not only how much total biomass was removed but also what component with a specific structure and from where it was removed. Identical biomass removal can result in very different substrate accessibility and therefore digestibility. A substrate with a small number of larger pores is more

accessible and digestible than a substrate with identical porosity (biomass removal) but with a large number of pores smaller than the molecular size of cellulase.

Glucan is a major component in lignocelluloses and the basic unit of cellulose; its removal by pretreatment is usually low and is often not used to explain cellulose accessibility. Under high severity pretreatment conditions, glucan removal can be high and will affect substrate porosity or accessibility. This is especially true for acid-based pretreatments with minimal lignin removal. It was found that substrate accessibility and enzymatic digestibility can be correlated to glucan solubilization as demonstrated in SO_2-catalyzed steam explosion experiments [56, 57, 72]. However, a very high glucan removal needed to achieve high cellulose saccharification is undesirable.

Physical Pretreatment/Substrate Size Reduction and Substrate/Cellulose Accessibility

Substrate size reduction is a physical pretreatment to increase substrate accessibility [73, 74]. Most studies in the literature used size reduction either prior to or post thermochemical pretreatment as an aid to thermochemical pretreatment but never with the intention as the sole means for cell wall deconstruction. This type of size reduction is often conducted to the level of fibers and fiber bundles without generating significant breakup of the cross-links between microfibrils within a fiber. We define this as Class I size reduction that does not significantly compromise the physical integrity of cell wall structure. In Class I size reduction as shown in Fig. 5a (1), fibers can be separated, cut, fragmented, and mildly externally fibrillated by shear forces to produce hairs or fibrils from the fiber surface which increase fiber external surface area—veryminor cell wall deconstruction [75, 76]. When conducted after an adequate thermochemical pretreatment, Class I size reduction can breakup cell wall with some level of fiber delamination [Fig. 5a (2)]. To better understand the effects of biomass size reduction on cellulose enzymatic saccharification and reconcile the conflicting results in the literature, we investigated a greater level of cell wall deconstruction defined as Class II size reduction. In Class II size reduction, biomass size is reduced to the level beyond fibers towards micro or even nanofibrils with significant breakup of microfibril cross-links and by compression-induced internal fibrillation [75–77]. Unlike in Class I, significant physical cell wall deconstruction takes place [Fig. 5b (1)], and the cell wall can be completely fibrillated to nanofibrills [Fig. 5b (2)] in Class II size reduction. The cell wall can also be significantly fragmented by external fibrillation [76, 78]. Ball milling is a typical Class II size reduction procedure. Recent studies using wet-disk milling to produce cellulose nanofibers or nanofibrils are other examples of Class 11 processes [78–81].

Class I size reduction has shown to be effective in increasing enzymatic saccharification of lignocelluloses especially for untreated substrates [12, 13]. However, the substrate size effects become less obvious for pretreated substrates [12, 24, 26]. This is because Class I mechanical size reduction to the degree of fibers or fiber bundles merely increases substrate external surface area without significantly deconstructing the cell wall to affect fiber pore structure. As discussed previously, substrate external surface area plays a minor role in contributing to overall saccharification, contributing approximately less than 10 %, comparing with internal pore surface area for pretreated substrates [38, 53]. Since most of the pores of untreated lignocelluloses are too small to be accessible to cellulase, the contribution of increasing external surface to saccharification of untreated lignocelluloses becomes critical and significant, but cannot achieve significant saccharification over 20 % [12, 13]. The contribution of external surface to saccharification is less important or negligible especially for adequately pretreated substrates with Class I size reduction. The observed minor positive effect of size reduction on pretreated substrates can be attributed to the increased external surface and minor cell wall deconstruction such as micorfibrils raised from the cell wall surface [Fig. 5a (1, 2)]. When applied prior to thermochemical pretreatment, Class I size reduction facilitates mass transfer such as chemical penetration in thermochemical pretreatment. When applied after thermochemical pretreatment such as in steam explosion or disk milling of pretreated woody biomass [24, 82] to reduce size reduction energy consumption [2, 83], it not only further exposes internal pores created by component removal during thermochemical pretreatment but also it can fragment, crack, and delaminate the cell wall of fibers to result in some level of cell wall deconstruction [Fig. 5a (2)], increasing substrate accessibility.

When size reduction proceeds beyond fiber level to microfibrils, i.e., Class II, significant physical destruction of the cell wall takes place, which simultaneously reduces the substrate crystallinity, a substrate reactivity parameter. Separating the effects of increased cellulose accessible area and reduced substrate crystallinity on saccharification is not possible at this level of size reduction. Although these two effects have very different mechanisms, they both are consequences of physical cell wall deconstruction, so we can use either a measure of size or crystallinity to represent the degree of cell wall deconstruction. One can expect that chemical pretreatment is completely unnecessary if the cell wall is completely deconstructed mechanically, i.e, to an amorphous state. This is supported by studies using ball milling of a pure cellulosic substrate [10, 84] as well as untreated softwood [84]. Using TEM,

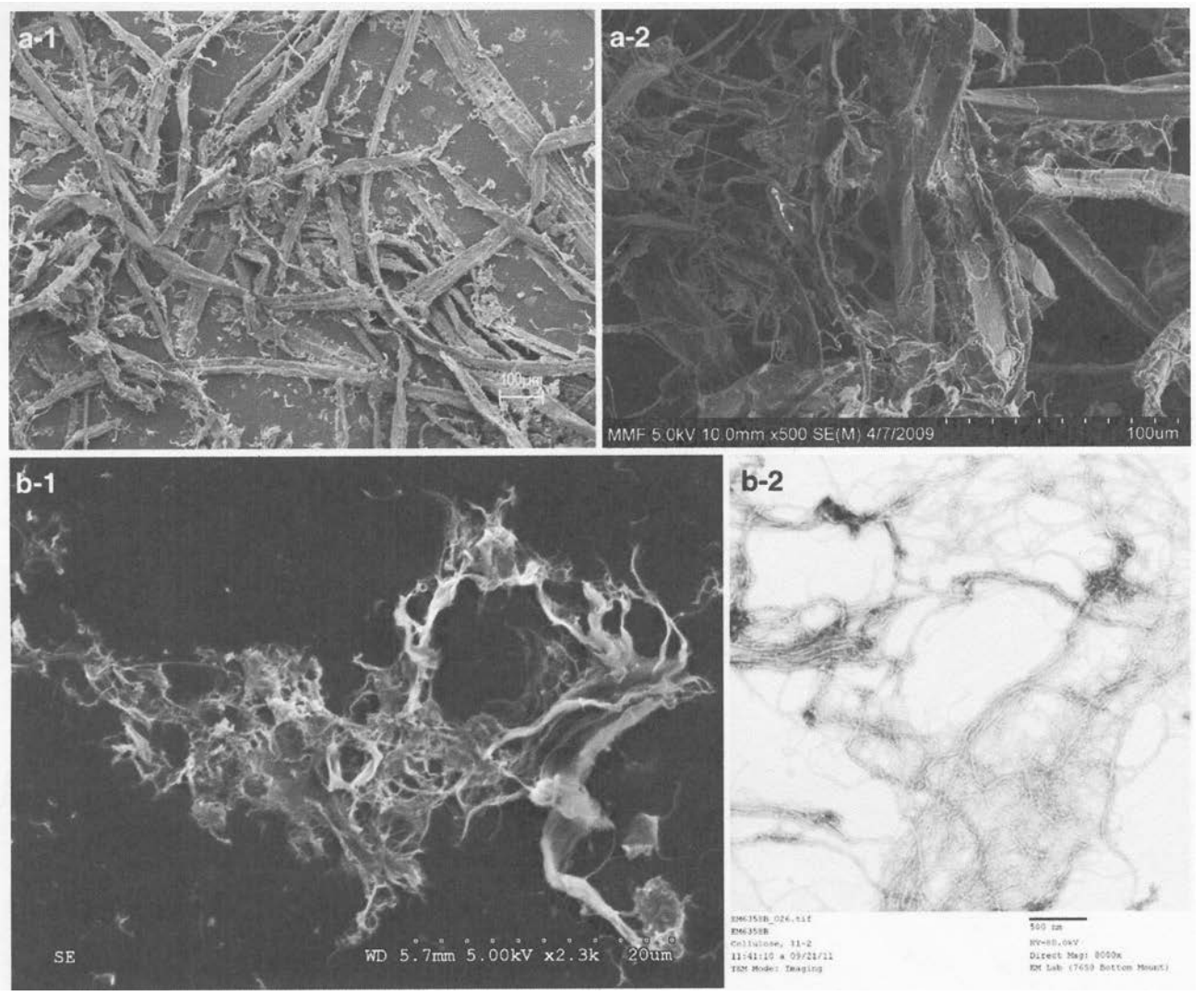

Fig. 5 Microscopic images of substrates produced by different level of size reduction. **a** *1* Class I size reduction with minimal fiber (cell wall) fibrillation: lodgepole pine pretreated by SPORL with sodium bisulfite charge 8 wt% at 180 °C for 30 min and disk milled with disk plat gap of 0.76 mm and discharged at 10 % solids consistency, energy input 0.9 MJ/kg; Substrate and data from [24]; **a** 2 Class I size reduction with breakup of cell wall and minor fiber delamination: lodgepole pine pretreated by SPORL with sulfuric acid and sodium bisulfite charge 2.2 and 8 wt%, respectively, at 180 °C for 30 min and disk milled with disk plat gap of 0.76 mm and discharged at 20 % solids consistency, energy input 0.5 MJ/kg; Substrate and data from [24]; **b** *1* Class II size reduction with deconstruction of cell wall: bleached eucalyptus pulp (Crystallinity Index=60.1) disk milled in a stone grinder for 5 h at 3 % consistency with energy input of 54.7 MJ/kg; Crystallinity Index= 45.4; b 2 Class II size reduction: TEM zoom into a region with complete deconstruction of cell wall to nanofibrills: bleached eucalyptus pulp disk milled in a stone grinder for 3 h at 3 % consistency with energy input of 32.0 MJ/kg; Crystallinity Index=46.0. Substrates and data from [81]. High heating value of wood ≈19 MJ/kg [2]

we observed gradual mechanical deconstruction of cell wall with time by using a special stone grinder to fibrillate wood fibers to different degrees of microfibril level [81]. Our unpublished data indicate that gradual cell wall deconstruction produced increased saccharification of untreated softwood to near complete saccharification. This suggests that mechanical deconstruction of the cell wall, i.e., Class II size reduction, renders complete accessibility of cellulose to result in near complete saccharification of lignocelluloses without any thermochemical pretreatment, even for very recalcitrant softwoods. However, energy consumption for mechanical size reduction increased significantly as indicated in Fig. 5 caption (comparing Fig. 5a (1) with b (1) and (2) in Fig. 5). Oftentimes, Class II size reduction to the level shown in Fig. 5b (1, 2) is not necessary. A hybrid approach, in which Class I and Class II size reduction levels overlap to achieve moderate cell wall deconstruction, can be used to compensate for mild or no chemical pretreatment to facilitate cellulose saccharification [35].

Component Inhibition to Affect Substrate Reactivity

Substrate reactivity is another important substrate-related factor important to lignocellulose saccharification. High crystalline cellulose has low reactivity with cellulase enzymes. For lignocelluloses, component inhibition is a major factor affecting substrate reactivity. Component inhibition refers to nonspecific (nonproductive) binding of cellulase to non-cellulosic components, such as lignin [3, 85–87], and hemicelluloses [88], which reduces effective enzyme activity. Most pretreatments result in two fractions of major cell wall components: the free (or unbound) fraction removed from the solid substrate and the bound fraction remaining on the solids. The inhibition of the unbound lignin fraction is often dealt with passively by washing the solid substrate [89] or using additives, However, washing consumes a significant amount of water [90]. Application of additives is another passive approach that has been used to address inhibition of primarily bound lignin [52, 70, 90–96]. Additives at the required dosage are expensive and also can cause potential problems. Further delignification to remove bound lignin was evaluated but is also expensive [97].

Recent studies improved the understanding of lignin binding to cellulase and the effect of lignin structure on cellulase binding [20–22, 98, 99]. These studies suggest that hydrophobic interaction is the primary driving force for cellulase binding to lignocelluloses [22]. Certain types of lignin are more inhibitive than others. For example, guaiacyl lignin usually has a slower reaction rate than syringyl type of lignin and more likely to be involved in condensation reactions [20, 71, 100]. Carboxylic acid groups tend to have low binding capacity to lignin [21]. Lignin sulfonation has proven to be favorable for efficient saccharification [99]. This is attributable to reduced nonspecific cellulase binding due to the presence of sulfonic acid groups that increased the hydrophilicity of lignin. The utility of this understanding is that lignin sulfonation can be achieved using sulfite-based pretreatments without separate post-treatment. This explains the effectiveness of SPORL processes for lignocellulose saccharification [55]. Furthermore, washing of SPORL pretreated substrates may be not necessary due to the low binding capacity of dissolved lignosulfonate to cellulase. As a matter of fact, a net gain in enzymatic saccharification can result when the whole slurry (the combined solid and liquor fractions of the pretreated lignocellulose) is enzymatically saccharified, as revealed in our recent study [101]. This is because dissolved lignosulfonate can act as a surfactant to offset its low inhibition effect due to its hydrophilic properties. This is a significant advantage in reducing water usage, reducing cellulase loading, as well as simplifying process integration.

Enzymatic Hydrolysis Conditions

Enzymatic hydrolysis conditions certainly affect cellulose saccharification. Optimal temperature and pH, the two main reaction environment parameters, have been optimized for a given enzyme formulation, i.e, at 50 °C and pH of 4.8–5.0, for most *Trichoderma reesi* cellulase based on laboratory cellulase activity measurements using pure cellulosic substrate (Product Sheet Celluclast 1.5 L, Novozymes). Almost all published work used this hydrolysis condition. pH can induce surface charge leading to altered surface hydrophobicity and therefore binding to cellulase. The surface charge can also affect electrostatic interactions and therefore binding between lignin and cellulase. This pH-induced surface charge varies with the substrate surface functional groups and substrate surface properties. Therefore, pH-induced surface property, e.g., hydrophobicity, variation will be different between cellulosic and lignocellulosic substrates and among different lignocelluloses. It is possible to use this difference to reduce nonspecific cellulase binding to lignin and to result in a net maximal cellulase binding to cellulose. This argument is corroborated by our recent study [101]. We found that the optimal pH range to achieve maximal cellulose saccharification for lignocellulosic substrates was between 5.2 and 6.2 [102], different from that for a pure cellulosic substrate, i.e, between 4.5 and 5.0 as suggested by cellulase manufacturers. The improvement in saccharification for SPORL pretreated lodgepole pine substrates can be increased by 80 %, from approximately 50 to 90 'Yo with a moderate cellulase loading of CTec2. Furthermore, the gain in SED by adjusting pH from 4.7 to 5.2 was found to correlate linearly to the lignin sulfonic group content [102].

Conclusions

Enzymatic saccharification of lignocellulloses can be analyzed using three basic processes of heterogeneous biochemistry reactions: (1) the intimate contact between the reactants — substrate accessibility to cellulase or CAC — and the roles of component removal and size reduction by thermo-chemical and physical pretreatments on this contact, (2) the reactivity between substrate and cellulase — limited by component inhibition, and (3) the reaction conditions — substrate-specific optimization. Removal of hemicelluloses is much more important than removal of lignin to create effective pores for improved CAC. Lignin coverage in the cell wall affects its ability to protect carbohydrate degradation and determines the relative importance between removal of lignin and hemicelluloses. Lignin removal is not important for low lignin content lignocelluloses. For high lignin content lignocelluloses, however, a minimal amount of lignin removal is required in addition to significant

or near complete hemicellulose removal to produce sufficient cellulose accessible surface to cellulase in order to achieve satisfactory cellulose saccharification. Substrate external surface plays a minor role in substrate accessibility. Substrate size reduction can be classified into two classes. Class I size reduction merely increases external surface without significant deconstruction cell wall and can only aid thermochemical pretreatment to produce a limited amount of additional saccharification. Class II size reduction significantly deconstructs the cell wall and produces a completely cellulase accessible substrate and therefore produces near complete saccharification without any other pretreatment.

Lignin structure and lignin surface functional groups have a significant impact on lignin nonspecific binding to cellulase. Lignin surface modification, especially through in-pretreatment modification such as sulfite-based processes, can be used to reduce lignin nonspecific binding. Adjusting hydrolysis pH to between 5.2 and 6.2 can result in a more hydrophilic lignin surface and favorable electrostatic interactions between lignin and cellulase enzymes to reduce lignin nonspecific binding, consequently a net gain in lignocellulose saccharification.

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