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3

Key features of pretreated lignocelluloses biomass solids and their impact on hydrolysis

R. KUMAR, Zymetis, Inc., USA and
C. E. WYMAN, University of California, USA

Abstract: Prior to biological conversion of lignocellulosic biomass to ethanol or other products, natural barriers developed to protect plants must be overcome to realize efficient enzymatic hydrolysis, and a few pretreatment technologies are effective in inexpensively accomplishing this task through heating with chemicals. Over the years, changes in a number of structural and compositional attributes of biomass have been postulated to explain how pretreatment enhances enzymatic hydrolysis performance, but the complexity of biomass has always confounded development of a unified theory that can unequivocally predict how pretreated biomass solids will respond to enzymes. However, sugar release can be viewed as ultimately governed by two factors: 1) access of enzymes to cellulose and hemicellulose and 2) the effectiveness of enzymes attached to the surface in breaking down these carbohydrate chains to sugars and/or their oligomers. In this review, this perspective of enzyme access and effectiveness is applied to findings reported in the literature to provide a framework for understanding how various features in pretreated biomass solids could affect deconstruction of cellulose and hemicellulose to sugars and their yields.

Key words: cellulase, cellulose, hemicelluloses, biomass, adsorption, accessibility, effectiveness, hydrolysis.

3.1 Introduction

Biological conversion of cellulosic biomass such as agricultural (e.g., corn stover) and forestry residues (e.g., sawdust) and herbaceous (e.g., switchgrass) and woody (e.g., poplar wood) energy crops into ethanol and other products offers the high yields to products vital to economic success, the potential for very low costs, and important strategic, environmental, and economic benefits (Farrell *et al.*, 2006; Gomez *et al.*, 2008; Lynd *et al.*, 1991, 1996, 1999; Ragauskas *et al.*, 2006; Schubert, 2006; Tilman *et al.*, 2006; Wyman, 1999, 2003; Zhang, 2008). However, cellulosic materials have developed a natural resistance to biological attack to assure survival (Dhugga, 2007; Himmel *et al.*, 2007), and a pretreatment step must be employed to overcome this resistance to high sugar yields (Chandra *et al.*, 2007; Grethlein, 1984; Lynd *et al.*, 2008;

Mosier *et al.*, 2005; Sun and Cheng, 2002; Weil *et al.*, 1994; Yang and Wyman, 2008). Dilute sulfuric acid is a leading option, but one economic study has projected it to be the most expensive single step in biomass conversion (Wooley *et al.*, 1999). A few other pretreatment technologies based on heating biomass with ammonia, pH buffers, lime, or sulfur dioxide give similar cost and performance to dilute sulfuric acid (Mosier *et al.*, 2005; Wyman *et al.*, 2005a, 2005b), but lower cost options are still needed. In addition, the significant repercussions of pretreatment for other processing steps must be fully considered in the choice of pretreatment (Yang and Wyman, 2008).

A more complete understanding of fundamental mechanisms responsible for pretreatment effectiveness would help accelerate development of lower cost approaches and improve their integration into the overall process (Wyman, 2007). Studies have attributed the effectiveness of pretreatment in improving enzymatic digestibility of biomass to increasing surface area and porosity (Chandra *et al.*, 2008; Grethlein, 1984, 1985; Ishizawa *et al.*, 2007; Mooney *et al.*, 1997, 1998; Tanaka *et al.*, 1988; Thompson *et al.*, 1992; Wong *et al.*, 1988; Zeng *et al.*, 2007), removal of hemicellulose and lignin (Grohmann *et al.*, 1986; Liu and Wyman, 2005; Pan *et al.*, 2005; Yang and Wyman, 2004; Zhu *et al.*, 2005), and reductions in cellulose crystallinity and the degree of polymerization (Chang and Holtzapple, 2000; Knappert *et al.*, 1980; Puri, 1984; Yoshida *et al.*, 2008). However, due to the complexity of biomass structures, changes in absolute cellulose crystallinity are difficult to determine accurately (Chang and Holtzapple, 2000; Puri, 1984; Puri and Pearce, 1986; Sun and Cheng, 2002). Interactions among other physical and chemical features of pretreated biomass make it difficult to isolate these variables and precisely determine which features have the greatest impact (Kumar *et al.*, 2009; Lynd, 1996; Mansfield *et al.*, 1999; Zhang and Lynd, 2004a). On top of that, enzymatic saccharification of cellulose is a heterogeneous reaction that requires successive adsorption of multiple enzymes on the surface for hydrolysis to occur (Kumar and Wyman, 2008; Lee and Fan, 1979; Ryu and Lee, 1982). These enzymes attain equilibrium with the substrate within an hour or two of incubation (Karlsson *et al.*, 1999; Kumar and Wyman, 2009b; Lynd, 1996), with the amount of adsorbed enzymes not changing significantly over the course of hydrolysis (Eriksson *et al.*, 2002b; Medve *et al.*, 1998; Xu *et al.*, 2008; Yu *et al.*, 1995), especially for lignocellulosic biomass. Cellulase adsorption is generally quantified by fitting parameters to the Langmuir isotherm equation (Lynd *et al.*, 2002; Walker and Wilson, 1991; Zhang and Lynd, 2004b), even though arguments have been made that this approach is oversimplified (Lynd *et al.*, 2002). Thus, the role of pretreatment in rendering biomass digestible for enzymes is unfortunately still ambiguous and not well understood.

In light of the complexity of biomass and the action of enzymes, we believe that enzymatic hydrolysis of cellulose and hemicellulose in pretreated biomass can be better viewed from the perspectives of the impact of substrate, enzyme,

and environmental chemical and physical factors on: 1) the accessibility of cellulose to enzymes, which is generally determined by the amount of enzyme adsorbed on cellulose in biomass, and 2) the effectiveness of the enzymes once they attach to cellulose. The emphasis of this review will be on identifying key substrate aspects that can impact enzyme adsorption or effectiveness or both based on information reported in the literature. Less detailed consideration will be given to important enzyme characteristics and physical parameters that likely impact these two potentially governing factors.

3.2 Key substrate features controlling cellulose hydrolysis: crystallinity

3.2.1 Accessibility

Enzymes are reported to rapidly hydrolyze amorphous cellulose to cellobiose and glucose, while the hydrolysis of crystalline cellulose is much slower, with the conclusion that the rate depends on cellulose crystallinity (Bertran and Dale 1985; Ghose and Bisaria, 1979; Wood *et al.*, 1989). The ordered structure of crystalline cellulose would impact the ability of cellulase to access cellulose based on the concept that a layer of cellulose must be removed before enzymes can reach layers (Fan *et al.*, 1980; Lee and Fan, 1983; Väljamäe *et al.*, 1999) and active sites lying underneath (Kongruang and Penner, 2004; Kongruang *et al.* 2004; Teeri, 1997; Zhang and Lynd, 2005), and studies that report rates slowing with increasing cellulose crystallinity are consistent with this hypothesis (Fan *et al.*, 1981; Sasaki *et al.*, 1979; Sinitsyn *et al.*, 1991). However, others have observed the opposite effect to be true: hydrolysis increases with crystallinity (Grethlein, 1985; Puri, 1984), though the results for real biomass may be misinterpreted because removal of amorphous lignin and/or hemicellulose would increase biomass crystallinity and enhance digestibility. Furthermore, if the hydrolysis rates are much slower for crystalline regions, a 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 (Ooshima *et al.*, 1983; Paralikar and Betrabet, 1977). However, no significant change in crystallinity has been measured over the course of cellulose hydrolysis (Boisset *et al.*, 1999; Chen *et al.*, 2007; Lenz *et al.*, 1990; Puls and Wood, 1991). In addition, in some cases, cellulose crystallinity was considered to have no effect on hydrolysis rates (Converse, 1993; Gharpuray *et al.*, 1981; Kim and Holtzapple, 2006; Mansfield *et al.*, 1999; Puri, 1984; Puri and Pearce, 1986; Rivers and Emert, 1988a, 1988b).

The following points may help address this conundrum and understand the mechanism better. First, recent studies suggest that cellulases not only function as an hydrolytic agent but can simultaneously disrupt the cellulose structure to a significant extent (Himmel *et al.*, 1999; Mansfield and Meder, 2003; Sinnot

1998; Wang *et al.*, 2008; Xiao *et al.*, 2001). Thus, during hydrolysis, the action(s) of individual monocomponent enzymes are likely offset by concurrent modification by complementing enzymes (Mansfield and Meder, 2003). Second, for real biomass, crystallinity should not be confused with absolute cellulose crystallinity as real biomass has amorphous components other than cellulose (Kim and Holtzapple, 2006; Kumar *et al.*, 2009). Third, use of high enzyme loadings to determine the impact of biomass features and other factors on hydrolysis may lead to misinterpretation by saturating the substrate. Fourth, almost all the characterization methods require a treatment before analysis such as drying, coating, etc., which may disturb the structure of the biomass. Nonetheless, better understanding of cellulases functioning at micro level and advanced analytical tools would help.

Cellulase adsorption could be a useful measure of changes in cellulose accessibility with crystallinity. The enzyme adsorption capacity of amorphous cellulose is much greater than for crystalline material, leading one to expect amorphous regions to have greater hydrolysis rates and yields (> 50 times) than for crystalline areas (Hong *et al.*, 2007; Jeoh *et al.*, 2007; Lynd, 1996; Meunier-Goddik and Penner, 1999; Ooshima *et al.*, 1983; Pinto *et al.*, 2006; Ryu and Lee, 1986; Sinitsyn *et al.*, 1991; Zhang and Lynd, 2004b, 2005). Cellulase adsorption capacity is generally quantified based on the following Langmuir equation:

$$[CE] = \frac{\sigma[S_t][E_f]}{K_d + [E_f]}$$

in which $[CE]$ is the amount of adsorbed enzyme in mg/g, $[E_f]$ the free enzyme concentration in mg/ml, σ the maximum adsorption capacity in mg/mg substrate, $[S_t]$ the substrate concentration in mg/ml, and K_d the equilibrium constant for the ratio $[C][E]/[CE]$ in mg of enzyme/ml. Representative values of the Langmuir parameters are summarized in Table 3.1 for a number of lignocellulosic materials reported in the literature; these parameters have been reviewed elsewhere for pure cellulose (Lynd *et al.*, 2002; Walker and Wilson, 1991; Zhang and Lynd, 2004b).

Unfortunately, most of the studies cited in Table 3.1 did not report cellulose/biomass crystallinity, so a clear trend could not be seen between crystallinity and adsorption capacity. But as reported elsewhere (Kumar, 2008; Kumar *et al.*, 2009) and discussed in the following sections, cellulose accessibility (as determined by cellulase adsorption) for a real biomass cannot be solely and clearly correlated with crystallinity. For example, Ooshima *et al.* reported that increasing pretreatment temperatures from 180 °C to 220 °C increased the adsorption capacity by almost six times, as shown in Table 3.1. This result would not be expected for pure cellulose because increased severity should remove more of the amorphous cellulose (Lenz *et al.*, 1990; Väljamäe *et al.*, 1999) and increase crystallinity, thereby making cellulose less accessible, as shown by Jeoh and corworkers (Jeoh *et al.*, 2007). However, the opposite was

Table 3.1 Langmuir parameters for lignocellulosic substrates for various enzymes and proteins

Substrate/source	Enzyme/ Protein/ Brand name	Max. Ads. Capacity σ, mg/g subs.	Affinity A, ml/mg protein	Ads. Strength $R = \sigma * A$, ml/g sub.	Reference
Birch, steam exploded	Celluclast 2L	214	2.1	42.8	Lee *et al.* (1994)
Birch, steam exploded and alkali extracted		237	2.8	663.6	
Wheat straw, unpretreated but cut, milled, and sieved < 0.177 mm	*Trichoderma* QM9414	8.34 (15 °C)	7.19	60.0	Estrada *et al.* (1988)
Wheat straw, NaOH pretreated		71.46 (15 °C)	2.27	162.3	
Delignified rice straw, lignin < 5%	*Trichoderma reesei*, D1-6	256	0.16	41.0	Goel and Ramachandran (1983)
Hardwood, dilute acid pretreated at 180 °C	*Trichoderma reesei*, GC 123, Genencor	14.1 (40 °C)	12.5	176.3	Ooshima *et al.* (1990)
Hardwood, dilute acid pretreated at 200 °C		30.5 (40 °C)	4.25	129.6	
Hardwood, dilute acid pretreated at 220 °C		80.6 (40 °C)	1.82	146.7	
Douglas Fir, steam exploded	Celluclast 2L + Betag-Novozyme 188	171.3	0.78	133.6	Lu (2002)
Douglas Fir, steam exploded alkali extracted		162.4	0.59	95.8	

Table 3.1 Continued

Substrate/source	Enzyme/ Protein/ Brand name	Max. Ads. Capacity σ, mg/g subs.	Affinity A, ml/mg protein	Ads. Strength $R = \sigma * A$, ml/g sub.	Reference
Corn stover, dilute acid pretreated at 190 °C	Cellulase, CPN from logen	60	nd		Kadam *et al.* (2004)
	Beta-g, Novozyme 188	10	nd		
Willow, steam pretreated	Celluclast 2L	471	0.29	136.6	Galbe *et al.* (1990)
Spruce, steam pretreated	CBHI	nd	5.5 (4 °C)		Palonen *et al.* (2004b)
	EGII	nd	1 (4 °C)		
Creeping wild ryegrass, dilute acid pretreated	Cellulase/ Celluclast 1.5 L	42.5	0.6	25.5	Zheng *et al.* (2007)
	Beta-g/ Novozyme 188	60.6	0.65	39.4	
Corn stover, dilute acid pretreated at 140 °C	Cellulase,	210 (4 °C)	6.36	1335.0	Willies (2007)
	Sp. CP	150 (50 °C)	0.221	33.2	
	Beta-g/	130 (4 °C)	0.09	11.7	
	Novozyme 188	140 (50 °C)	0.09	12.6	
	BSA	30 (4 °C)	0.078	2.34	
		130 (50 °C)	0.175	22.75	
Ethanol pretreated lodgepole pine (EPLP), lignin ~14.5%	Spezyme CP	60.35	3.17	190	Tu *et al.* (2007)
	Celluclast	87.69	3.48	310	
Steam exploded lodgepole pine (SELP), lignin ~ 45.6%	Celluclast	101.05	1.48	150	
Hardwood, dilute acid pretreated at 220 °C	C. thermocellum	317 (60 °C)	344	10.9E+4	Bernardez *et al.* (1993)
Corn stover solids					
AFEX[a]	Genencor	99.7	1.86	185.4	Kumar and Wyman (2009b)
ARP	Spezyme CP	113.8	46.2	5257.6	
C. pH	(@ 4 °C)	101.7	0.77	78.3	
D. acid		90.7	2.49	225.8	
Lime		133.6	0.88	100.0	
SO_2		124.8	0.90	112.3	
Poplar solids					
AFEX	Genencor	107.4	0.21	23.0	Kumar and Wyman (2009a)
ARP	Spezyme CP	113.5	0.11	13.1	
C.pH	(@ 4 °C)	56.2	0.43	23.9	
D.acid		170.9	0.94	159	
Flowthrough		195.2	0.08	15.6	
Lime		150.8	0.09	14.5	
SO_2		142.2	1.14	161.0	

[a] Pretreatment type ; AFEX – ammonia fiber expansion; ARP – ammonia recycled percolation; C.pH – controlled pH; D. acid – dilute acid.

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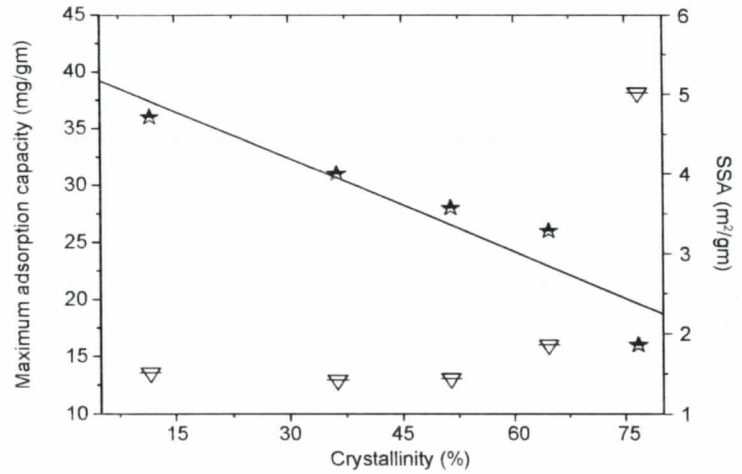
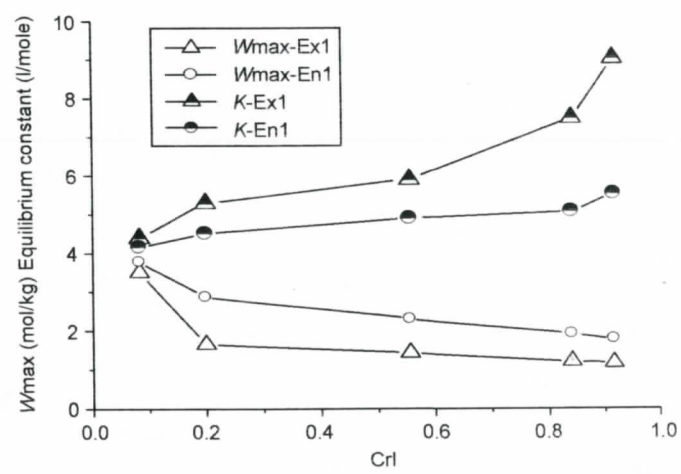


3.1 Effect of cellulose crystallinity on maximum cellulose adsorption capacity (Lee *et al.*, 1980). SSA is the specific surface area.

observed because increasing temperature (severity) not only makes possible changes in cellulose crystal structure but removes hemicellulose (Grethlein, 1985). In addition, cellulose DP is reduced (Knappert *et al.*, 1980; Kumar *et al.*, 2009), and lignin-hemicellulose/cellulose bonds are no doubt ruptured (Gupta *et al.*, 2008; Kumar *et al.*, 2009).

For pure cellulosic substrates, Lee and coworkers (Lee *et al.*, 1982) reported a decline in adsorption capacity of cellulose for complete cellulases[1] with increasing crystallinity, as shown in Fig. 3.1. Although the specific surface area (SSA in Fig. 3.1) did not increase as crystallinity dropped, typically SSA and crystallinity are related to solids produced by mechanical pretreatments. Ooshima *et al.* documented similar cellulase adsorption patterns at 5 °C for cellulose of varying crystallinity prepared by enzymatic digestion of Avicel cellulose for different times (Ooshima *et al.*, 1983). Similarly, Hoshino *et al.* showed that purified exo and endo cellulases of *Irpex lecteus* had an inverse correlation between cellulose crystallinity and the maximum amount of protein adsorbed, as shown in Fig. 3.2 (Hoshino and Kanda, 1997; Hoshino *et al.*, 1992). In a kinetic study, Ryu *et al.* demonstrated an increase in adsorption kinetic parameters with a drop in crystallinity (Ryu and Lee, 1986). In another study, Sinitsyn and coworkers (1991) reported an inverse correlation between crystallinity of pure cellulose and the adsorption of peroxidase and chymotrypsin proteins on cellulose. However, for baggase, protein adsorption was shown to increase with

1. Throughout the chapter complete cellulose(s) refers to the crude mixture containing two cellobiohydrolases (CBHI and CBHII), five endoglucanases (EG I to EG V), and a β-glucosidase, unless otherwise stated.



3.2 Effect of cellulose crystallinity on maximum adsorption capacity and equilibrium constants for exo and endocellulase (Hoshino *et al.*, 1997). W_{max} is the maximum amount of enzyme adsorbed and K is the equilibrium constant.

delignification and a reduction in crystallinity index (CrI). For sodium hydroxide pretreated wheat straw, Estrada and coworkers (Estrada *et al.*, 1988) found an inverse correlation between adsorption parameters and crystallinity.

A study of cellulose binding domains and cellulose interaction showed greater adsorption of binding domains to amorphous than to crystalline cellulose (Pinto *et al.*, 2006). Recently, Joeh and coworkers (Jeoh *et al.*, 2007) revealed that crystallinity greatly reduces adsorption of Cellobiohydrolase I (Cel7A; CBHI), leading to a decreased extent of hydrolysis. Furthermore, air drying of dilute acid pretreated corn stover resulted in a decrease in the extent of CBHI adsorption, probably due to 'hornification' of fibers (Esteghlalian *et al.*, 2001) and/or increased crystallinity due to drying (Weimer *et al.*, 1995).

Different cellulase components have different adsorption capacities and activities (Lynd, 1996; Zhang and Lynd, 2004b), as shown in Table 3.2, where Avicel is highly crystalline (CrI = ~60%) and has a shorter cellulose chain length (DP ~ 300) than filter paper (CrI = ~40%; DP ~ 750–2800) (Zhang *et al.*, 2006). Endoglucanse-I, which attacks and adsorbs preferentially on amorphous cellulose, was measured to have an average adsorption capacity and activity greater than for CBH-I on both types of cellulose studied. A similar pattern for Endoglucanase I (EGI, Cel7B) was reported by Ding and Xu (2004), but Klyosov (1982) observed that the adsorption capacity of endoglucanses from *Trichoderma reesei* did not depend on cellulose crystallinity. Yet, contrary to the numerous studies mentioned above, working with pure cellulose and lignocellulosic substrates, Goel and Ramachandran (1983) found no correlation

Table 3.2 Adsorption capacity for cellulase components and their activity on cellulose substrates

Parameter	Substrate							
	Avicel				Filter paper			
	Temperature/enzyme component				Temperature/enzyme component			
	Temp (°C)	CBH-I	Temp (°C)	EG-I	Temp (°C)	CBH-I	Temp (°C)	EG-I
Maximum adsorption capacity (mg/g or μmol/g)	20 25 4 20 40 30	69 70 48 51.8 40 63	30	126	50	*0.17*	50	*0.17*
Avg.		**57**		**126**		**0.17**		**0.17**
Specific activity (μmol glucose Equiv./mg/min)	50 45 40 30	0.065 0.04 0.012 0.019	50 45 40 30	0.045 0.17 0.0046 0.196	50 50 40	0.08 0.22 0.0046	50 50 40	0.18 1.2 0.0023
Avg.		**0.034**		**0.104**		**0.102**		**0.461**

*The data shown above were adapted from Lynd *et al.* (2002) and Zhang and Lynd (2004b).

between crystallinity and adsorption of cellulase enzymes activities. Furthermore, Banka *et al.* showed that adsorption of a non-hydrolytic protein designated Fibril Forming Protein (FFP) from *Trichoderma reesei* increased with crystallinity (Banka and Mishra, 2002).

3.2.2 Effectiveness

In addition to accessibility, cellulose crystallinity would likely impact the effectiveness of adsorbed cellulase components. The literature shows that cellulose crystallinity affects the synergism between cellulase components (Henrissat, 1994; Henrissat *et al.*, 1985; Hoshino and Kanda, 1997; Hoshino *et al.*, 1997; Kanda *et al.*, 1980; Murashima *et al.*, 2002; Nidetzky *et al.*, 1993; Tarantili *et al.*, 1996; Väljamäe, 2002; Väljamäe *et al.*, 1999; Zhang and Lynd, 2004b). Hoshino *et al.* found increased synergism between CBHI and Endoglucanase II (EGII) from *T. ressei* with increased crystallinity and determined the highest synergism between Cellobiohydrolase II (CBHII, Cel6B) and EGII to be for a crystallinity index ~ 1.0. In another study, Igarashi and coworkers showed that the nature of the crystalline cellulose polymorph also affected hydrolytic activity of adsorbed Cel7A; for example, 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 (Igarashi *et al.*, 2006a, 2006b, 2007). Moreover, Mizutani *et al.* (2002) and Gama and Mota (1997) showed that the beneficial impact of surfactant on saccharification is influenced by crystallinity for pure cellulose. However, there is evidence that the presence of surfactants helps reduce unproductive adsorption of enzymes not only on lignin but on cellulose as well (Eriksson *et al.*, 2002a; Kumar and Wyman, 2009d; Ooshima *et al.*, 1986) and thus enhances their effective activity. Furthermore, several 'restart studies' with pure microcrystalline cellulose have shown that unproductive binding of enzymes is one of the main reasons for the slow down of hydrolysis rate over hydrolysis time (Kumar and Wyman, 2009c; Ooshima *et al.*, 1991; Yang *et al.*, 2006). Besides, Ma and coworkers in a recent study have shown that irreversibly surface bound CBHI loses up to 70% of its activity in just 10 minutes (Ma *et al.*, 2008). On a different note, Gruno *et al.* reported that end-product inhibition of cellulase was higher for crystalline cellulose than amorphous (Gruno *et al.*, 2004). Therefore, it appears that crystallinity impacts enzyme effectiveness.

A more limited literature indicates that the processivity of the dominant enzyme of the *Trichoderma* system, Cel7A (CBHI) is affected by cellulose crystallinity. A rough estimate of processivity as measured in terms of the ratio of cellobiose to glucose released from bacterial microcrystalline cellulose (BMCC, CrI ~ > 85 %) and amorphous cellulose was reported to be 23 and 14, respectively, by Ossowski and coworkers (von Ossowski *et al.*, 2003). In another

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study, these processivity measures for *Trichoderma reesei* Cel7A were reported to be 88 ± 10, 42 ± 10, and 34 ± 2.0 cellobiose units for bacterial cellulose (BC, CrI ~ 88), bacterial microcrystalline cellulose (BMCC, CrI ~ 92), and endoglucanse-pretreated bacterial cellulose (unknown CrI), respectively (Kipper *et al.*, 2005). Although the study offered no explanation, the low processivity values for BMCC and endoglucanase treated BC could be due to hindrance by solitary chains left after erosion of the surface by the enzyme, unproductively adsorbed enzymes, and/or the nature of the substrate (Henrissat, 1998; Nutt *et al.*, 1998; Väljamäe *et al.*, 1999). Overall, because of the importance of enzyme action to hydrolysis rates, further studies are needed to elucidate the role of crystallinity on the processivity of Cel7A and other processive or pseudo-processive enzymes from various micro-organisms (Horn *et al.*, 2006).

Per theoretical models, it is believed that crystalline cellulose chains have some structural irregularities that provide attack sites for endoglucanses (Lynd *et al.*, 2002; Teeri, 1997). However, the literature leads us to believe that some cellulase components bound to this amorphous fraction of cellulose, which would be smaller in size for highly crystalline cellulose, may not be very active due to their large size, compared to the length of the amorphous fraction of cellulose microfibrils. In particular, the 35–40 cellobiose lattices occupied per bound cellulase molecule (Hong *et al.*, 2007; Zhang and Lynd, 2004b) and/or their slow catalytic reaction rates due to the low processivity of CBHI (von Ossowski *et al.*, 2003) limit the effectiveness of endo-glucanases by covering their preferential active sites. Incomplete hydrolysis of amorphous cellulose by Cel7A reported by Joeh *et al.* (2007) and competition among cellulase components during adsorption (Kyriacou *et al.*, 1989; Nidetzky *et al.*, 1996; Nieves *et al.*, 1991; Ryu *et al.*, 1984), mostly studied for microcrystalline cellulose, strongly support this idea. Furthermore, a nonlinear correlation was observed between CBHII adsorption and activity on filter paper (CrI ~ 40%) by Nidetzky *et al.* (1994a), and EGI was found to be more active compared to CBHI and CBHII, even though the maximum binding capacity of EGI was roughly equal to that for CBHI and much lower than for CBHII. In another study, Eriksson *et al.* found that although Cel7A (CBHI) unproductively adsorbed on steam-pretreated spruce (SPS), it desorbed back into solution when supplemented with Cel7B (EGI), enhancing conversion, perhaps due to preferential attack of amorphous cellulose by EGI releasing unproductively adsorbed CBHI (Eriksson *et al.*, 2002b).

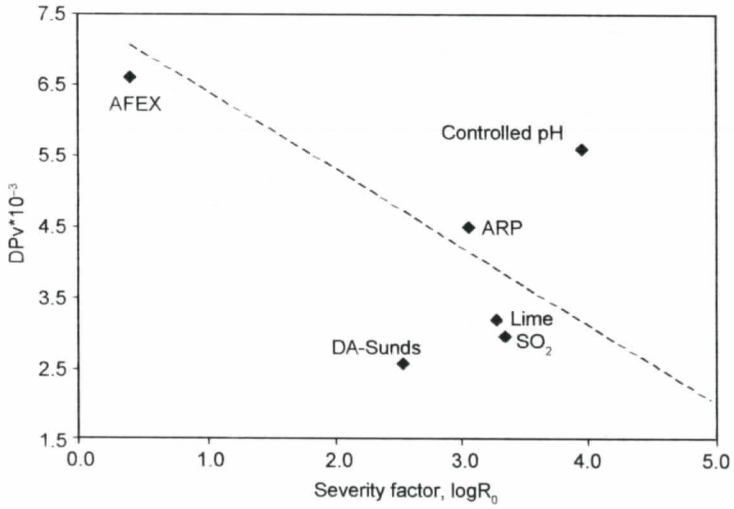
3.3 Key substrate features controlling cellulose hydrolysis: degree of polymerization (DP)

Several studies and literature reviews discuss the change in DP of insoluble and soluble cellulose during and after hydrolysis by complete cellulase mixtures or mono-components (Cao and Tan, 2002; Converse, 1993; Eremeeva *et al.*, 2001;

Hilden *et al.*, 2005; Kanda *et al.*, 1976; Kleman-Leyer *et al.*, 1996; Mansfield and Meder, 2003; Mansfield *et al.*, 1999; Martinez *et al.*, 1997; Pala *et al.*, 2007; Zhang and Lynd, 2004b, 2005). However, the understanding of the impact of cellulose chain length on hydrolysis is still limited, and questions about cellulose DP and what role, if any, cellulose chain length plays in cellulose hydrolysis are still unanswered. Among the very few studies on this subject, Puri and coworker (Puri, 1984; Puri and Pearce, 1986) showed that a reduction in cellulose DP improved hydrolysis, but a lack of data on the effect on surface area and other substrate features makes conclusions of this study inconclusive. In another study, Knappert and coworkers developed a qualitative relationship between cellulose DP and digestibility (Knappert *et al.*, 1980). Sinistyn and co-researchers showed that reduction in DP of cotton linters by γ-irradiation, while keeping crystallinity index (CI) constant, had a negligible impact on hydrolysis rates (Sinitsyn *et al.*, 1991). A recent kinetic study by Zhang and Lynd indicated that a decrease in cellulose DP had a less effect on accelerating hydrolysis rates than increasing the accessibility of β-glycosidic bonds as measured by the maximum amount of cellulase adsorbed on cellulose (Zhang and Lynd, 2006). However, the possibility of how cellulose chain length (DP) may affect accessibility was not discussed.

3.3.1 Accessibility

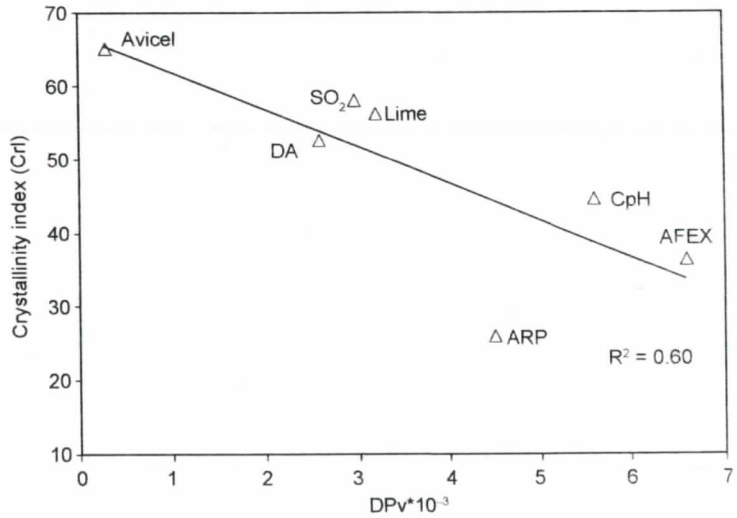
Given the typically large amount of CBHI in cellulase (>65%) and its catalytic site preferences (Beldman *et al.*, 1985; Christina Divne, 1998; Nidetzky *et al.*, 1994b; Teeri, 1997; Teeri *et al.*, 1995), one could conclude that DP reduction should improve hydrolysis effectiveness by making more reducing chain ends available to CBHI (and non-reducing ends available to CBHII, which is about 20% of the total cellulase protein), with the result that lowering DP would be a promising target to enhance cellulose accessibility. However, cellulose crystallinity and DP appear to be closely correlated for mechanical pretreatments and the majority of thermochemical methods such as steam explosion and dilute acid, making it difficult to differentiate which controls (Chandra *et al.*, 2007; Kumar *et al.*, 2009). For example, mechanical pretreatments such as ball milling generally reduce both crystallinity and DP (Caulfield and Moore, 1974; Lee *et al.*, 1982; Oh and Kim, 1987; Schwanninger *et al.*, 2004; Sinitsyn *et al.*, 1991) but could also affect lignin structure in real biomass, making it difficult to isolate the effect of just cellulose DP on enzyme adsorption and hydrolysis. Increasing thermochemical pretreatment severity removes a substantial portion of the amorphous region (Kumar *et al.*, 2009), increasing substrate crystallinity, but the cellulose chain length may also drop to the level off degree of polymerization (LODP) (Håkansson and Ahlgren, 2005; Heitz *et al.*, 1987; Kumar *et al.*, 2009; Martinez *et al.*, 1997; Millett *et al.*, 1954; Treimanis *et al.*, 1998). The relationship of viscosity average degree of polymerization (DP_v) to



3.3 Effect of pretreatment severity on cellulose degree of polymerization (DPv) as measured by the viscosity method for corn stover solids prepared by leading pretreatment technologies. The line is shown to help follow the trend but is not fit to the data. AFEX – ammonia fiber expansion, ARP – ammonia recycled percolation, DA – dilute acid, CpH – controlled pH, SO_2 – sulfur dioxide (Kumar, 2008; Kumar *et al.*, 2009).

pretreatment severity[2] (log R_0) is shown in Fig. 3.3 for corn stover solids prepared by leading pretreatment technologies that all employ heating with chemicals (Mosier *et al.*, 2005; Wyman *et al.*, 2005b), and DP drops with severity for almost all of these options. However, crystallinity[3] can also be related to DP for several pretreatments, as shown in Fig. 3.4, clouding the interpretation of this data due to the drop in cellulase adsorption with increasing crystallinity discussed before. In another study, Engstrom and coworkers found that pulp's accessibility and reactivity for the viscose process increased significantly following treatment with monocomponent endoglucanases, which also resulted in DP reduction; however, similar results, at a comparable DP level, were not observed when pulp was treated with acid (Engstrom *et al.*, 2006). Although the information on the effect of cellulose DP on cellulase adsorption is limited, Kaplan and coworkers (Kaplan *et al.*, 1970) showed a significant drop in cellulase adsorption and associated lower hydrolysis of altered cellulose following photochemical degradation, which was probably due to a decrease

2. Severity factor, defined as $R_0 = t \cdot \exp[(T_H - T_R)/14.75]$, includes only time and temperature parameters; however, all of these pretreatments except controlled pH (CpH) utilize different chemicals at various concentrations.
3. Crystallinity values were adopted from Laureano-Perez *et al.* (2005).



3.4 Crystallinity vs. cellulose viscosity degree of polymerization for corn stover solids prepared by leading pretreatment technologies. AFEX – ammonia fiber expansion, ARP – ammonia recycled percolation, DA – dilute acid, CpH – controlled pH, SO_2 – sulfur dioxide (Kumar, 2008).

in cellulose DP and some ring opening for weathered cotton cellulose. Yet, it is a well known quoted fact that 80% of fungal cellulase protein (CBHI and CBHII) preferably attacks chain ends (Carrard and Linder, 1999; Henrissat *et al.*, 1985; Teeri *et al.*, 1995), but unfortunately almost nothing has been done to conclusively show the impact of cellulose DP on cellulase adsorption.

3.3.2 Effectiveness

Theoretically, the lower the DP, the more reducing and non-reducing ends are available, and one would expect that more CBHI/II would be able to work at one time while making it easier for endoglucanases to act. For soluble cellulose, Nidetzky *et al.* found that the initial degradation velocity of cello-oligosaccharides by CBHI increased with DP below cellohexose and then remained constant for higher DP (Nidetzky *et al.*, 1994b). Similar effects of DP for soluble cellodextrins on CBHII and EGI activity are reviewed elsewhere (Zhang and Lynd, 2004b). Furthermore, a decrease in β-glucosidase activity with increasing DP has been reported (Lee and Fan, 1980; Wilson *et al.*, 1994). However, to the authors' knowledge, no information is available on the effect of insoluble cellulose DP on the catalytic efficiency of cellulase except that higher DP could result in higher synergy between CBHI and EGI (Henrissat, 1994; Okazaki *et al.*, 1981; Okazaki and Moo-Young, 1978; Zhang and Lynd, 2006). Furthermore, cellulose DP may affect the processivity index, with full processivity of

CBHI possibly not realized for short chains (Gupta and Lee, 2009; Väljamäe *et al.*, 1999). 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 the differences in their cellulose DP and crystallinity (Puri, 1984; Ramos *et al.*, 1993), likely due to the complexity of real cellulosic substrates.

3.4 Key substrate features controlling cellulose hydrolysis: hemicellulose and degree of hemicellulose acetylation

3.4.1 Accessibility

It has been postulated that hemicellulose impedes access to cellulose by forming a sheath around glucan chains (Berlin *et al.*, 2007; Ding and Himmel, 2006; Himmel *et al.*, 2007; Jeoh *et al.*, 2007; Kumar and Wyman, 2009f, 2009g; Selig *et al.*, 2008; Yoshida *et al.*, 2008), and several studies showed a direct relationship between cellulose digestion and hemicellulose removal (Allen *et al.*, 2001; Grohmann *et al.*, 1986; Ishizawa *et al.*, 2007; Jeoh *et al.*, 2005; Kabel *et al.*, 2007; Kim *et al.*, 2001; Palonen *et al.*, 2004a; Um *et al.*, 2003; Yang and Wyman, 2004; Zhu *et al.*, 2005), with some even concluding that lignin removal is not necessary for good cellulose conversion (Clark *et al.*, 1989; Grohmann *et al.*, 1986). However, some substrates required high temperatures for the same degree of hemicellulose removal to be effective, suggesting that hemicellulose removal is not the only factor impacting digestibility (Torget *et al.*, 1991; Yang *et al.*, 2004). In addition, some reports do not postulate any role for hemicellulose removal in changing cellulose digestibility (Fan *et al.*, 1982; Millett *et al.*, 1975; Tsao *et al.*, 1978). Unfortunately, hemicellulose alteration can also disrupt other biomass components (Chum *et al.*, 1988; Grethlein, 1984; 1985; Iyer and Lee, 1999; Kumar *et al.*, 2009; Maloney *et al.*, 1985), making it challenging to draw firm conclusions about the degree to which it controls access of enzymes to cellulose. In addition, some contend that hemicellulose may actually be a marker related to disruption of the far less soluble lignin and that lignin disruption could be the key to greater digestion (Liu and Wyman, 2003, 2004a, 2004b, 2005; Yang and Wyman, 2004).

Less attention has been given to how the degree of acetylation of the substrate impacts cellulose digestion. Hemicellulose chains are extensively acetylated in many types of biomass, and deacetylation was reported to enhance cellulose digestibility significantly, with some differences noted in the degree of removal needed (Kim and Holtzapple, 2005; Kumar and Wyman, 2009e; Lemos *et al.*, 2000; Wood and McCrae, 1986). Removing hemicellulose also removes acetyl groups (Kabel *et al.*, 2007; Maloney *et al.*, 1985) and usually alters the form of lignin (Ooshima *et al.*, 1990; Selig *et al.*, 2007) left on the material, making it

difficult to isolate which factor was most influential in improving performance. One study showed that this effect appeared to become less important beyond removal of 75% of the acetyl groups (Grohmann *et al.*, 1989), while other studies demonstrated continual improvements up to full removal (Kong *et al.*, 1992; Kumar and Wyman, 2009e, 2009f). Grohmann and coworkers showed that removing acetyl esters from aspen wood and wheat straw made them 5 to 7 times more digestible, and Kong and coworkers (1992) observed a major effect of removing the acetyl content of aspen wood on cellulose digestibility even though lignin and polysaccharides were left in place. Consistent with this, Kumar and Wyman observed a significant enhancement in glucan (from 17 to ~40%) and xylan (from 6% to ~30%) digestibility by selective removal of acetyl groups from corn stover (Kumar and Wyman, 2009f), and > 60% of glucan and xylan digestion was realized with further supplementation of xylanase to cellulase. However, Chang and Holtzapple (2000) applied similar methods to poplar wood as above but showed that removal of acetyl bonds is less important than reduction in crystallinity and/or removal of lignin.

Unfortunately, it is still debatable whether hemicellulose removal or the breakdown of cross-linked network of polysaccharides and bonds among them is responsible for enhanced digestion of cellulose in pretreated biomass. For example, Weimer and coworkers (2000) suggested that intimate association of xylan and cellulose does not inhibit the bio-degradability of polysaccharides. Furthermore, from a more applied perspective, some pretreatments such as Ammonia Fiber Expansion (AFEX) produce highly digestible cellulose without removing much hemicellulose (Dale *et al.*, 1996; Teymouri *et al.*, 2005; Vlasenko *et al.*, 1997) but remove acetyl groups and probably other side chains from xylan, disrupting linkages among carbohydrates and lignin to a significant extent (Chundawat *et al.*, 2007; Kumar *et al.*, 2009). Although the role of acetyl groups and other side chains removal may seem limited, it is pretty clear that removal of these side chains during pretreatment would surely result in the reduction of enzyme requirements and enhance both xylan and glucan digestions as well (Fernandes *et al.*, 1999; Kumar and Wyman, 2009f; Selig *et al.*, 2008).

Although its role in enhancing cellulose digestion is ambiguous, hemicellulose/xylan removal during pretreatment may be desirable for economic and technical reasons such as higher recovery of xylose and less need for hemicellulose degrading and accessory enzymes (Hespell *et al.*, 1997; Knauf and Moniruzzaman, 2004; Kumar and Wyman, 2009g; Merino Sandra and Cherry, 2007). In addition, in a recent study we showed that removing hemicellulose during pretreatment can reduce cellulase/xylanase inhibition by soluble xylo-oligomers generated during enzymatic hydrolysis (Kumar and Wyman, 2009e, 2009f). Similarly, Kim *et al.* showed that the effluent exiting from Ammonia Recycled Percolation (ARP) pretreatment of corn stover; containing mostly xylooligomers, soluble lignin, and sugar and lignin degradation products, inhibited cellulase and microbial activity significantly (Kim *et al.*, 2006).

Furthermore, Suh and Choi showed that xylooligomers inhibited endo-xylanase action (Suh and Choi, 1996).

We believe that even slight branching of hemicellulose and its acetylated network can interfere with cellulase access to cellulose (Karlsson *et al.*, 2002; Pan *et al.*, 2006; Samios *et al.*, 1997; Yu *et al.*, 2003), but this is difficult to prove in that direct information on the effect of acetylation and hemicellulose on cellulose accessibility is scarce. However, Jeoh and coworkers (2005, 2007) recently reported increased cellulose accessibility, as measured by the adsorption of fluorescent labeled Cel7A (CBHI), and an increase in hydrolysis with the extent of xylan removal. It is also reported in several recent studies that supplementation of cellulase with xylanase, which should selectively only remove xylan, not only enhanced xylan conversion but glucan digestion as well. In addition, the linear relationship generally found between xylan and glucan digestion basically indicates that xylan removal affects cellulose accessibility (Berlin *et al.*, 2007; Beukes *et al.*, 2008; García-Aparicio *et al.*, 2007; Gupta *et al.*, 2008; Kumar and Wyman, 2009f, 2009g; Murashima *et al.*, 2003; Selig *et al.*, 2008). Hemicellulose deposition on cellulose during pretreatment (Gray *et al.*, 2003, 2007; Kumar and Wyman, 2009f; Linder *et al.*, 2003; Nagle *et al.*, 2002) could also reduce the amount of cellulose available for cellulase action. Pan *et al.* in a study suggested (Pan *et al.*, 2006) that acetyl groups in pulp may restrict cellulase accessibility to cellulose by inhibiting productive binding through increasing the diameter of cellulose and/or changing its hydrophobicity. Selective deacetylation of corn stover by the Kong *et al.* method (Kong *et al.*, 1992) enhanced CBHI adsorption significantly more than delignification, increased the initial rate, and produced greater digestibility of cellulose and xylan as well, indicating increased cellulose accessibility (Kumar and Wyman, 2009b, 2009f). However, not much information is available in the literature to clarify whether selective hemicellulose removal and/or deacetylation impacts cellulase adsorption/accessibility, and further study is needed to understand the impact of xylooligomers on cellulase (xylanase) adsorption.

3.4.2 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 (Anand and Vithayathil, 1996; Fernandes *et al.*, 1999; Glasser *et al.*, 1995; Grabber *et al.*, 1998a; Grohmann *et al.*, 1989; Kormelink and Voragen, 1992; Mitchell *et al.*, 1990; Rivard *et al.*, 1992; Shallom and Shoham, 2003; Suh and Choi, 1996; Tenkanen *et al.*, 1996; Wood and McCrae, 1986), which in turn increases cellulose digestibility (García-Aparicio *et al.*, 2007; Kumar and Wyman, 2009a, 2009b; Murashima *et al.*, 2003; Tabka *et al.*, 2006; Yu *et al.*, 2003). Although the effect of xylan removal

on cellulase efficiency is not yet known, it presumably affects the processiv action of Cel7A by binding cellulase unproductively (Chernoglazov *et al.*, 1988 Tenkanen *et al.*, 1995) and, as discussed earlier, xylan oligomers, release during hydrolysis and pretreatments, strongly inhibit enzymes activity (Kim *et al.*, 2006; Kumar and Wyman, 2009c, 2009e, 2009g; Suh and Choi, 1996 Although the direct effect of acetyl groups on cellulase effectiveness, howeve may not yet be clear, they certainly affect xylanase effectiveness, as shown b Kumar and Wyman (Kumar and Wyman, 2009b, 2009f). Some literature report further lead us to believe that acetylated/substituted xylooligomers should b much more inhibitory to enzymes effectiveness than just plain xylooligome (Kumar and Wyman, 2009e; Suh and Choi, 1996), as removal of acetyl group substitution from soluble xylooligomers by means of hydrolytic action accessory enzymes such as acetyl xylan esterase and L-arabinofuranosidas would facilitate break down of xylooligomers by xylanase and beta-xylosidas and consequently would have lesser impact on cellulase action. Thus, more wor is needed to clarify whether hemicellulose removal and deacetylation impact th accessibility of cellulase to cellulose or the effectiveness of cellulase o cellulose or both.

3.5 Key substrate features controlling cellulose hydrolysis: lignin

3.5.1 Accessibility

Lignin binds cellulosic fibers together in a composite structure with excelle properties but also shields cellulose from accessibility to enzymes (Wyman *al.*, 2005b). Various studies reported cellulose hydrolysis was improved wi increasing lignin removal, although differences were reported in the degree lignin removal needed (Converse, 1993; Grethlein, 1984; Yang *et al.*, 200 Yang and Wyman, 2004). Besides the degree of lignin removal, the ratio syringyl to guaiacyl moieties in the lignin was considered to significant influence digestibility (Yamamoto *et al.*, 1990).

Overall, the protective lignin sheath is thought to present a major impedime to enzymatic hydrolysis of cellulose in pretreated biomass by restricting enzyn accessibility to cellulose (Chandra *et al.*, 2007; Chapple *et al.*, 2007; Mansfield *al.*, 1999; Pan *et al.*, 2005; Saddler *et al.*, 1982; Taniguchi *et al.*, 2005). T majority of studies in the literature have reported that enzymatic conversion polysaccharides is enhanced by delignification of hardwood/softwood and lign cellulosics (Chang and Holtzapple, 2000; Cunningham *et al.*, 1981; Gharpuray *al.*, 1981; Kabeya *et al.*, 1993; Koullas *et al.*, 1993; Liao *et al.*, 2005; Morrisc 1983; Sawada *et al.*, 1995; Schwald *et al.*, 1988a; Stinson and Ham, 1995; Su *et al.*, 1976; Yu *et al.*, 1998); however, others found none or a negati correlation between lignin content/removal and digestibility of residual cellulo

(Draude *et al.*, 2001; Jeoh *et al.*, 2005; Kim *et al.*, 2001; Saddler *et al.*, 1982; Wong *et al.*, 1988).

Overall, the exact role of lignin in limiting hydrolysis has been difficult to define. One of its most significant effects is on fiber swelling and the resulting influence on cellulose accessibility (Mooney *et al.*, 1998; Nelson and Oliver, 1971). For example, Yuldashev *et al.* observed that the amount of cellulase on the surface of cotton stalks (cellulose – 44%, lignin – 26.4%) was lower than for milled cotton stalks (cellulose – 92%, lignin – 0.6%), leading to a drop in conversion; however, lignin did not inactivate free or bound enzyme (Yuldashev *et al.*, 1993). In another study, Ishihara and coworkers determined that lignin slows down enzyme adsorption but does not restrict carbohydrate conversion for steamed shirakamba wood (Ishihara *et al.*, 1991). Limited delignification of wheat straw by sodium hydroxide, though not selective, was shown to increase cellulase adsorption by Estrada *et al.* (1988). Conversely, Mooney *et al.* concluded that the proportion of lignin does not influence cellulase adsorption for four different types of pulp that differed in lignin content (Mooney *et al.*, 1997). Although several studies suggested that lignin removal/or lignin content does not affect cellulase adsorption on cellulose/biomass significantly (Eriksson *et al.*, 2002a; Lu *et al.*, 2002; Mooney *et al.*, 1998), it has rarely been shown experimentally whether selective lignin removal affects cellulase adsorption. For the first time, Kumar and Wyman showed that selective removal of lignin from corn stover did not significantly increase cellulase accessibility to cellulose, as measured by purified Cel7A adsorption. Instead, lignin removal appeared to more directly affect xylan accessibility, which in turn affected cellulose accessibility, as evidenced by a much higher increase in xylan digestion than glucan and a linear relation between the percentage increase in xylan and glucan conversions (Kumar and Wyman, 2009b). Consistent with this hypothesis, in another study, we found that lignin removal by the acid chlorite method from biomass solids pretreated with high pH pretreatments, which leave most of the xylan in place, resulted in much higher enhancement of glucan and, especially, xylan digestibility, compared to low pH pretreatments such as dilute acid and SO_2 steam explosion, which are known for their effectiveness in removing most of the hemicellulose during pretreatment (Kumar and Wyman, 2009a). Öhgren *et al.* also found a negligible impact of delignification on glucan digestibility of steam exploded corn stover (Öhgren *et al.*, 2007). Furthermore, consistent with the above findings, Selig and coworkers reported that lignin appears to have a more direct impact on xylan than glucan accessibility by purified cellulase and xylanase activities, which in turn occludes glucan accessibility (Selig *et al.*, 2009). Several studies in previous years reported that lignin removal affects hemicellulose more than glucan hydrolysis (Beveridge and Richards, 1975; Ford, 1983; Mes-Hartree *et al.*, 1987; Morrison, 1983; Prabhu and Maheshwari, 1999; Teixeira *et al.*, 1999). For example, Chang and coworkers applied lime pretreatment to effectively remove lignin from switchgrass with a 5 and 21 times increase in glucan and xylan digestibility, respectively (Chang *et al.*, 1997). On a different note, Mes-Hartree and coworkers employing biologically delignified aspen wood (BDA; 44% lignin removal) and steamed aspen wood for cellulase production showed that *Trichoderma harzianum* produced a low level of cellulase and gave significantly lower sugar yields for BDA than steamed aspen wood, because the latter had fewer pentosans than BDA and delignification did not result in enhanced cellulose accessibility (Mes-Hartree *et al.*, 1987).

Lignin has been claimed to depolymerize, dissolve, repolymerize, and then precipitate during pretreatment by hemicellulose hydrolysis, although no doubt in a different morphology that could change its impact on cellulose digestion (Donohoe *et al.*, 2008; Li *et al.*, 2007; Ramos *et al.*, 1993; Schell *et al.*, 1991; Schwald *et al.*, 1988b; Selig *et al.*, 2007; Shevchenko *et al.*, 1999; Yang and Wyman, 2004). In addition, there is evidence that the high solubility of hemicellulose could aid in taking lignin into solution despite the low solubility of the later (Gray *et al.*, 2003, 2007), but that the lignin would fall back onto the biomass once it breaks free from hemicellulose and polymerizes to low solubility compounds (Liu and Wyman, 2003, 2004a, 2004b, 2005; Yang *et al.*, 2004). The removal/disruption of lignin may not only increase accessibility of xylan and cellulose, though indirectly, but also make more cellulase and other enzymes available to act (Kumar and Wyman, 2009d; Yang and Wyman, 2004). Because lignin is physically and chemically resistant to attack by enzymes, irreversiblly absorbs cellulase (and other enzymes), and acts as a impenetrable barrier to cellulase, its presence limits xylan/cellulose accessibility (Kumar and Wyman, 2009a, 2009b; Lu *et al.*, 2002).

Adsorption of enzymes/proteins on lignin has been shown to follow a Langmuir isotherm, with typical parameters shown in Table 3.3. The unproductive binding of protein to lignin is dependent on the source and its preparation (Kumar and Wyman, 2009a, 2009b; Ooshima *et al.*, 1990; Sutcliffe and Saddler, 1986) and could likely be reduced by using additives (Boerjesson *et al.*, 2007; Eriksson *et al.*, 2002a; Sewalt *et al.*, 1997a; Tu *et al.*, 2007; Yang and Wyman, 2006). For example, as shown in Table 3.3, we found that lignin residues enzymatically extracted from corn stover and poplar solids, prepared by leading pretreatment options, had different cellulase adsorption capacities and affinities. Surprisingly, lignin prepared by dilute acid pretreatment, at least for poplar, had a low cellulase adsorption capacity but lignin prepared with AFEX pretreatment was found to have the least. It appears that chemicals/reagents used in pretreatment significantly affect lignin characteristics as there was no direct relationship found between pretreatment temperature/severity (log R_0; includes time and temperature only) and adsorption parameters. However, Ooshima *et al.* applied dilute acid pretreatment of hardwood to show a decline in adsorption capacity with an increase in temperature due to shrinking and agglomeration of lignin (Ooshima *et al.*, 1990). Similar observations of lignin melting and its

Table 3.3 Langmuir parameters for enzyme/protein adsorption on lignin

Substrate/source	Enzyme/ Protein/ Brand name	Max. Ads. Capacity σ, mg/g subs.	Affinity A, ml/mg protein	Ads. Strength $R = \sigma * A$, ml/g sub.	Reference
Larch lignin	EGI	–	0.09	–	Chernoglazov *et al.* (1988)
	EGII	–	0.11	–	
Beech lignin	EGI	–	0.03	–	
	EGII	–	0.03	–	
Lignin residue/180 °C*	Cellulase GC 123, Genencor	100	0.41	40.8	Ooshima *et al.* (1990)
Lignin residue/200 °C		66.6	0.66	43.6	
Lignin residue/220 °C		12.3	0.81	9.93	
EL[1]	Celluclast 1.5 L	86.1	0.51	43.9	Zheng *et al.* (2007)
	Beta-g/Novo 188	173.5	0.75	129.8	
EL[2]	Cellulase, Sp. CP	590 (4 °C)	0.06	37.8	Willies (2007)
		790 (50 °C)	0.18	140	
	Beta-g/Novo188	170 (4 °C)	0.45	76.5	
		130 (50 °C)	0.86	112	
	Bovine Serum Albumin	180 (4 °C)	0.64	115	
		280 (50 °C)	0.91	255	
Alkali lignin	CBHI/CBHI-CD/ EGII/EGII-CD	–	1.7/0.0/ 0.6/0.2	–	Palonen *et al.* (2004b)
EL[3]		–	0.6/0.0/ 0.2/0.0	–	
Corn stover-enzyme lignin[2]					
AFEX[a]	Cellulase, Spezyme CP	38.7	2.99	116.0	Kumar and Wyman (2009b)
ARP		41.6	10.70	445.0	
C. pH		63.6	0.60	36.2	
D. acid		53.0	0.68	174.5	
Lime		64.9	2.69	37.8	
SO_2		67.5	6.39	431.5	
Poplar-enzyme lignin[2]					
AFEX	Cellulase, Spezyme CP	56.8	2.14	121.8	Kumar and Wyman (2009a)
ARP		92.1	0.59	54.8	
D. acid		74.0	0.29	21.2	
FT		112.8	0.67	75.9	
Lime		126.9	0.11	14.3	
SO_2		83.7	0.25	21.0	
Alkali-lignin	Xylanase, Pulpzyme HC	–	11.8 (pH 4.0)/ 8.9 (pH 9.0)	–	Ryu and Kim (1998)

* Lignin was obtained from dilute acid pretreated hardwood prepared at three different temperatures. There is no information if the remaining protein was completely dislodged from lignin surface.

1. Lignin was obtained after complete enzymatic hydrolysis of carbohydrate part of dilute acid pretreated creeping wild rye grass. There is no information if the protein left on lignin was removed before adsorption studies.
2. Lignin was obtained after complete enzymatic hydrolysis of carbohydrate part (< 15% of carbohydrate left in substrate) of dilute acid pretreated corn stover. The protein remaining on lignin residue was dislodged by protease treatment.
3. Lignin was obtained after complete enzymatic hydrolysis of carbohydrate part of steam pretreated softwood. It was reported that 5.5% protein was left adsorbed on lignin after washing.

[a] Pretreatment type; AFEX – ammonia fiber expansion; ARP – ammonia recycled percolation; C.pH – controlled pH; D. acid – dilute acid.

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relocation are affirmed by others as well (Donaldson *et al.*, 1988; Donohoe *et al.*, 2008; Michalowicz *et al.*, 1991; Selig *et al.*, 2007). In a recent study, Selig *et al.* explained that droplets of lignin, formed during high temperature dilute acid or water only pretreatment, migrate to the cell wall, and may deposit on the cellulose surface to impede cellulase adsorption on cellulose (Selig *et al.*, 2007).

Lignin removal is expensive, and it is not clear whether lignin removal or disruption of its tight association with carbohydrates is more important. Grabber and coworkers suggested that inhibition of fungal hydrolases is not affected by lignin composition (Grabber *et al.*, 1997); however, lignin concentration and its cross-linking with feruloylated xylans greatly affect degradability of cell wall (Grabber, 2005; Grabber *et al.*, 1998b). Yet, a negative impact of lignin concentration on cell wall digestibility of tobacco stems was observed by Sewalt and coworkers (1997b) in another study.

3.5.2 Effectiveness

Although lignin's effect on hydrolysis is not entirely clear, lignin removal is technically and economically advantageous prior to cellulose saccharification because unproductive binding to lignin reduces enzyme availability, thereby limiting cellulase effectiveness (Berlin *et al.*, 2005, 2006; Excoffier *et al.*, 1991; Jørgensen and Olsson, 2006; Kumar and Wyman, 2009a; Mandels and Reese, 1965; Selig *et al.*, 2007; Sewalt *et al.*, 1997a; Wu and Lee, 1997; Yang and Wyman, 2006), lignin breakdown products are likely to be inhibitory to fermentation and cellulase effectiveness (Hartley *et al.*, 1976; Kaya *et al.*, 1999; Lynd, 1996), and lignin increases viscosities (Berson *et al.*, 2006; Fan *et al.*, 2003) at the higher solid loadings needed commercially (Wingren *et al.*, 2003), requiring more energy and negatively affecting cellulase effectiveness (Jørgensen *et al.*, 2007; Nutor and Converse, 1991; Pimenova and Hanley, 2003; Väljamäe *et al.*, 2001). Furthermore, lignin and its derivatives were also reported to precipitate and bond with protein (Kawamoto *et al.*, 1992; Makkar *et al.*, 1987). In addition, during pretreatment, some soluble lignin depolymerization and degradation compounds may form, and these compounds, though their impact on cellulase adsorption is not known, may severely inhibit enzyme effectiveness (Excoffier *et al.*, 1991; García-Aparicio *et al.*, 2006; Kaya *et al.*, 1999; Paul *et al.*, 2003; Selig *et al.*, 2007; Weil *et al.*, 2002). Literature studies suggest that lignin droplets deposited on cellulose may interact with water, as one study shows that hydrophobic surfaces at a macroscopic level do not repel but attract water (van Oss, 1995), and form a boundary layer impeding cellulase movement (Donohoe *et al.*, 2008; Matthews *et al.*, 2006; Selig *et al.*, 2007). Unproductive cellulase adsorption on lignin is hypothetically considered due to hydrophobic interactions (Bai *et al.*, 2008; Kongruang *et al.*, 2003; Tilton *et al.*, 1991). In some studies, the extent of hydrolysis and the amount of free enzyme have been reported to increase with increased cellulase hydrophilicity (Kajiuchi

et al., 1993; Park *et al.*, 2002), because proteins are highly hydrophobic due to clusters of closely located non-polar residues on their surface (Andreaus *et al.*, 1999; Halder *et al.*, 2005; Karlsson *et al.*, 2005; Reinikainen *et al.*, 1995; Suvajittanont *et al.*, 2000) and tend to adsorb strongly on hydrophobic surfaces (Kongruang *et al.*, 2003; van Oss, 1995). Furthermore, protein attachment to highly hydrophobic surfaces results in conformational changes and consequently irreversible adsorption and deactivation (Borjesson *et al.*, 2007; Kajiuchi *et al.*, 1993; Palonen, 2004; Park *et al.*, 2002). In addition, lignin linkages with cellulose (Jin *et al.*, 2006; Karlsson and Westermark, 1996; Kotelnikova *et al.*, 1993) presumably impact the processive action of cellulase. Although lignin may reduce the active amount of enzyme available for cellulose hydrolysis, its relationship to effectiveness of adsorbed cellulase still needs further study.

3.6 Conclusions

Overall, it can be concluded that literature reports on enzymatic hydrolysis of cellulose can be viewed in terms of two key factors, cellulase accessibility to cellulose and cellulase effectiveness. For example, several studies have shown a strong correlation between rates/extent of hydrolysis and enzyme adsorption (Beltrame *et al.*, 1982; Ding *et al.*, 2000; Hogan *et al.*, 1990; Karlsson *et al.*, 1999; Klyosov, 1986; Kotiranta *et al.*, 1999; Lee and Fan, 1979, 1982; Mansfield *et al.*, 1999; Medve *et al.*, 1998; Mooney *et al.*, 1999; Nidetzky and Steiner, 1993; Sakata *et al.*, 1985; Sethi *et al.*, 1998; Watson *et al.*, 2002; Yang *et al.*, 2006), and we recently observed an almost linear relationship between the maximum protein adsorption capacity of cellulase on solids and the hydrolysis rate and yield in a study with corn stover and poplar solids prepared by promising pretreatment technologies (Kumar, 2008; Kumar and Wyman, 2009b; Mosier *et al.*, 2005).

Although cellulase accessibility to cellulose appears to be affected more by xylan removal than lignin removal, cellulase adsorption and its efficacy cannot be related to a solitary substrate feature or two for lignocellulosics. As summarized in Table 3.4, other substrate features may also have a significant impact on the two factors hypothesized to primarily control hydrolysis; however, the extent of their impact may either be lower than xylan/lignin removal or unclear due to their interdependence with lignin/xylan removal. For example, cellulose crystallinity appears to significantly impact accessibility, at least as suggested for cellulase adsorption data for pure cellulose and for pretreatments using reagents such as phosphoric acid that generate amorphous cellulose (Zhang *et al.*, 2007). However, conventional methods used to determine biomass crystallinity may suggest otherwise. In addition, even for other thermochemical pretreatments, the reagents used in combination with heat not only disrupt lignin-carbohydrate linkages but change hydrogen bonds among cellulose chains

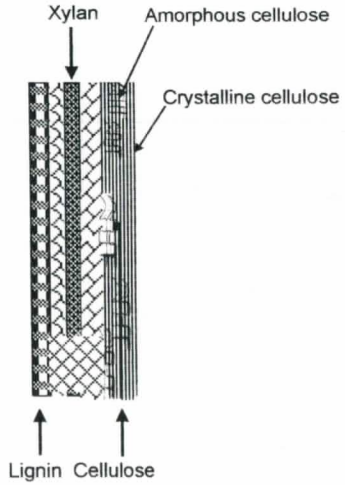
Table 3.4 A summary of how primary substrate features are hypothesized to impact cellulase accessibility to cellulose and cellulase effectiveness with impact ranking

Substrate features	Cellulase accessibility to cellulose (Impact ranking[a])	Cellulase effectiveness (Impact ranking)
Acetyl groups	Small but noticeable effect (02)	Yes (04)
Cellulose crystallinity	Yes[b]	Yes[b]
Cellulose DP	Inconclusive	Largely inconclusive but some impact (01)
Xylan content	A significant impact (10)	A major impact (06)
Lignin removal	Appears negligible (0.5)	A significant impact (08)

[a] Ranking was based on 0 to 10, where 10 stands for the highest impact on the feature noted and zero for negligible impact.
[b] Ranking was not given due to lack of convincing resolution in literature.

as well (Chundawat *et al.*, 2007; He *et al.*, 2008; Kumar *et al.*, 2009). Consequently pretreated lignocellulosic solids, in most cases, have much higher cellulose accessibility (Kumar and Wyman, 2009a, 2009b), resulting in higher digestibility than pure cellulose such as Avicel (Kumar and Wyman, 2009e; Lloyd and Wyman, 2005). Thus, the role of crystallinity in cellulose accessibility remains unclear. For example, although the origins are different and cellulase effectiveness may differ, bacterial cellulose (BC; CrI ~ 60 to 70%) and bacterial microcrystalline cellulose (BMCC CrI ~> 85%) both have similar or higher crystallinity but much higher accessibility than microcrystalline cellulose Avicel (CrI ~ 50 to 60%) (Hong *et al.*, 2007; Zhang and Lynd, 2004b).

The literature also suggests that lignin does not directly limit glucan accessibility but greatly restricts xylan accessibility which in turn limits glucan accessibility, as shown by a simplified conceptual model in Fig. 3.5. According to this model, lignin is strongly linked to xylan but also has bonds to glucan, whereas xylan is more strongly linked to glucan than lignin and functions as a filler or spacer between lignin and glucan layers. Therefore, either xylan or lignin removal should enhance saccharification, but because xylan removal directly impacts glucan chain accessibility, removing xylan should be more advantageous than removing lignin. In addition to direct impact on enzyme accessibility to glucan, xylan removal has some additional advantages; for example, xylan removal should result in 1) reduced enzyme inhibition by xylooligomers and 2) reduced requirements for xylanases and other auxiliary enzymes for xylan debranching. However, lignin removal exposes more xylan, resulting in the need for additional xylan degrading and auxiliary enzymes to expose glucan to cellulose and makes more enzymes available for hydrolysis due to reduced unproductive binding. In addition, removing lignin during pretreatment could have a big impact on process

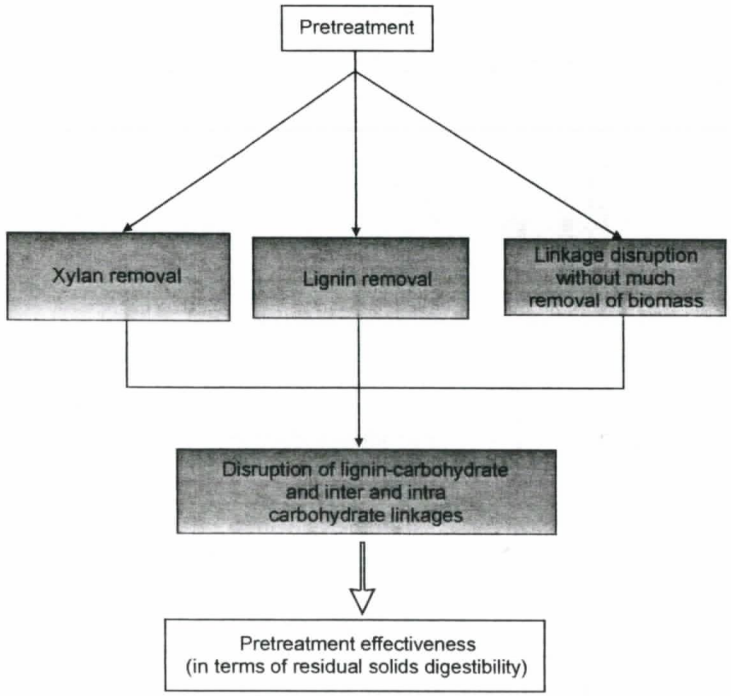


3.5 A simplified conceptual model of biomass structure.

economics by lowering mixing requirements in fermentation and making lignin available for other uses, provided lignin removal costs are low.

Ammonia fiber expansion (AFEX) pretreatment is unique in that although AFEX removes little lignin or xylan, it still gives good digestibility, at least for non woody biomass. This anomaly could be attributed to disruption of lignin-carbohydrate linkages (LCC) (Chundawat *et al.*, 2007; Kumar *et al.*, 2009; Laureano-Perez *et al.*, 2005; Venkatesh *et al.*, 2009) and lignin alteration resulting in reduced affinity for enzymes (Kumar and Wyman, 2009a, 2009b). Thus, based on cellulose accessibility (Kumar and Wyman, 2009a, 2009b) and hydrolysis data with AFEX (Sendich *et al.*, 2008; Venkatesh *et al.*, 2009), it could be concluded that LCC disruption is the most important requirement for an effective pretreatment, as shown in Fig. 3.6, with spacer (xylan)/lignin removal merely a way to accomplish this goal.

Overall, altering the substrate through reducing substrate hemicelluloses, lignin, and acetyl contents; crystallinity; and degree of polymerization can particularly affect accessibility of enzymes to cellulose. However, although changes in the substrate can be necessary to realize good enzyme effectiveness, they may not be sufficient because of the importance of the nature of the numerous cellulase components and chemical and physical environmental factors to performance. For example, once cellulase protein adsorbs on the surface, its catalytic efficacy may further be dictated by physical parameters such as pH, temperature, ionic strength, and the presence of inhibitors (Andreaus *et al.*, 1999; Kumar and Wyman, 2008; Panagiotou and Olsson, 2007; Reinikainen *et al.*, 1995; Tengborg *et al.*, 2001) as well as factors related to the substrate and enzyme.



3.6 A schematic decision tree of pretreatment effectiveness.

Although the focus of this review is on how modifications in biomass affect enzymatic hydrolysis, cellulase components molar ratios and their concentrations may affect their adsorption and effectiveness due to synergistic action (Beukes *et al.*, 2008; Gupta *et al.*, 2008; Murnen *et al.*, 2007; Selig *et al.*, 2008), and supplementation of cellulase with other enzymes such as β-glucosidase/xylosidase, xylanase, and debranching enzymes may also enhance cellulase adsorption/effectiveness, depending on substrate and pretreatment type (Girard and Converse, 1993; Huang and Penner, 1991; Väljamäe *et al.*, 2001). The physical and chemical environment, substrate loadings (Kumar and Wyman, 2008; Stutzenberger and Lintz, 1986; Xiao *et al.*, 2004), sugars (Kristensen *et al.*, 2009; Kumar and Wyman, 2008; Todorovic *et al.*, 1987; Wendorf *et al.*, 2004), their oligomers (García-Aparicio *et al.*, 2006), sugar degradation products (Kaya *et al.*, 1999; Sineiro *et al.*, 1997), chemical compounds (Eriksson *et al.*, 2002a; Park *et al.*, 1992), additives (Kim *et al.*, 1988; Moloney and Coughlan, 1983), temperature (Golovchenko *et al.*, 1992; Reinikainen *et al.*, 1995), pH (Gerber *et al.*, 1997; Kim and Hong, 2000), ionic strength (Azevedo *et al.*, 2000; Sakata *et al.*, 1985), and agitation (Azevedo *et al.*, 2000; O'Neill *et al.*, 2007; Sakata *et al.*, 1985) have all been hypothesized to play roles in influencing

enzyme accessibility and effectiveness. On this basis, a concerted effort is needed to better understand fundamental physical and chemical features of lignocellulosic biomass that limit its deconstruction and the organization and interaction among biomass components that constitute a barrier to access by enzymes to breakdown carbohydrates into fermentable sugars. Such an understanding of factors that control the interactions of substrates and enzymes would be invaluable in identifying pathways to lower cost advanced coupled pretreatment and enzymatic hydrolysis systems. Because new accurate data are critical to meaningfully assess promising advances in plant, microbial, and enzymatic systems, improved analytical methods must also be developed to fully characterize biomass composition and its structure and characterize interactions among biomass and various chemical treatments, as well as with deconstruction and hydrolysis enzymes.

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