ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Strong Cellulase Inhibition by Mannan Polysaccharides in Cellulose Conversion to Sugars

Rajeev Kumar,[1,2] Charles E. Wyman[1,2,3]

[1]Center for Environmental Research and Technology (CE-CERT), Bourns College of Engineering, University of California, Riverside, 1084 Columbia Avenue, Riverside, California 92507; telephone: +951-781-5668; fax: +951-781-5790; e-mail: rkumar@cert.ucr.edu

[2]BioEnergy Science Center (BESC), Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6422

[3]Department of Chemical and Environmental Engineering, Bourns College of Engineering, University of California, Riverside, California

ABSTRACT: Cellulase enzymes contribute a major fraction of the total cost for biological conversion of lignocellulosic biomass to fuels and chemicals. Although a several fold reduction in cellulase production costs and enhancement of cellulase activity and stability have been reported in recent years, sugar yields are still lower at low enzyme doses than desired commercially. We recently reported that hemicellulose xylan and its oligomers strongly inhibit cellulase and that supplementation of cellulase with xylanase and β-xylosidase would significantly reduce such inhibition. In this study, mannan polysaccharides and their enzymatically prepared hydrolyzates were discovered to be strongly inhibitory to fungal cellulase in cellulose conversion (>50% drop in % relative conversion), even at a small concentration of 0.1 g/L, and inhibition was much greater than experienced by other known inhibitors such as cellobiose, xylooligomers, and furfural. Furthermore, cellulase inhibition dramatically increased with heteromannan loading and mannan substitution with galactose side units. In general, enzymatically prepared hydrolyzates were less inhibitory than their respective mannan polysaccharides except highly substituted ones. Supplementation of cellulase with commercial accessory enzymes such as xylanase, pectinase, and β-glucosidase was effective in greatly relieving inhibition but only for less substituted heteromannans. However, cellulase supplementation with purified heteromannan specific enzymes relieved inhibition by these more substituted heteromannans as well, suggesting that commercial preparations need to have higher amounts of such activities to realize high sugar yields at the low enzyme protein loadings needed for low cost fuels production.

Biotechnol. Bioeng. 2014;111: 1341–1353.

Correspondence to: Rajeev Kumar

Contract grant sponsor: Office of Biological and Environmental Research in the DOE Office of Science through the BioEnergy Science Center (BESC)

Received 15 December 2013; Revision received 24 January 2014; Accepted 6 February 2014

Accepted manuscript online 12 February 2014;

Article first published online 1 May 2014 in Wiley Online Library (http://onlinelibrary.wiley.com/doi/10.1002/bit.25218/abstract).

DOI 10.1002/bit.25218

KEYWORDS: cellulase; inhibition; heteromannans; glucomannan; galactomannan; oligomers; xylan

Introduction

Lignocellulosic biomass comprised of three major components—cellulose, hemicellulose, and lignin- can sustainably be converted into biofuels to help meet world needs for transportation fuels and address global climate change and other mounting environmental concerns (Lynd et al., 1991; Wyman, 1994). However, its recalcitrant nature poses challenges to biological conversion to fuels at high yields for the low enzymes protein loadings needed to keep commercial costs low (Lynd et al., 1999, 2008). In addition, many of the end-products of enzyme action severely inhibit enzyme action and result in low sugar yields. For example, Mandels, Reese, and coworkers pointed out inhibition by glucose in the 1950s (Mandels and Reese, 1957; Reese, 1957), Halliwell and Griffin reported substantial inhibition by cellobiose in the 1970s (Halliwell and Griffin, 1973), and our team recently showed severe inhibition by xylan oligomers (Kumar and Wyman, 2009b,c).

The most frequently studied cellulase from the filamentous fungi *Trichoderma reesei* is comprised of several activities including two cellobiohydrolases (CBH I/Cel 7A; CBH II/Cel 6A), five endoglucanases (EG I/Cel 7B, EG II/Cel 5A, EG III/Cel 12A, EG IV/Cel 61 A, and EG V/Cel 45A), and at least two β-glucosidases (βGL 1/Cel 3A; βGL 2/Cel 1A) (Bayer et al., 1998; Henrissat et al., 1985). Most of these components contain two domains—a catalytic domain and a cellulose binding domain (CBD)—that are connected by *o*-glycosylated peptide linkers. Through a heterogeneous reaction, cellobiohydrolases I and II aided by endoglucanases processively deconstruct insoluble cellulose chain from reducing and non-reducing ends, respectively, to produce mostly cellobiose and small amounts of glucose and

cellotriose (Stahlberg et al., 1991, 1993). The reaction end-products cellobiose and glucose in turn inhibit cellulase catalytic activity (Gong et al., 1977; Gusakov et al., 1985; Holtzapple et al., 1990; Hong et al., 1981) as well as adsorption (Kumar and Wyman, 2008). The negative effect of sugars on cellulase adsorption as pointed out by (Kumar and Wyman, 2008) was later confirmed by (Kristensen et al., 2009), and is believed to be one of the main causes for drop in conversion at high solids loading. However, β-glucosidases hydrolyze cellobiose and higher soluble cellooligomers via a homogenous reaction into glucose, thereby reducing inhibition of cellobiohydrolases and endoglucanases (Gong et al., 1977; Hong et al., 1981; Kadam et al., 2004).

Although recent advances have been reported to enhance cellulase specific activity and stability and dramatically reduce production costs (Zhang et al., 2006, 2012), the enzyme loadings required to achieve high sugar yields are still high and expensive, particularly at the high biomass solids loadings favored commercially (Di Risio et al., 2011). For example, Klein-Marcuschamer et al. (2012) recently reported that the cost contribution of enzymes to ethanol production from corn stover can be $0.68/gallon even at theoretical maximum sugar yields. One of the reasons for such high enzyme costs is their strong inhibition by plant derived inhibitors. For instance, as pointed out earlier, we recently showed that xylan, a major hemicellulose constituent that comprises up to 90 wt% of total hemicellulose in most hardwood species, energy crops, and agricultural residues, and its shorter chain fragments commonly called xylooligomers (XOs) strongly inhibit enzyme activities (Kumar and Wyman, 2009b,c). In another study, Qing et al. (2010) showed that XOs were stronger inhibitors than cellobiose at equal molar concentrations. Kont et al. (2013) also showed that the oligosaccharides of xylan and mixed linkages β-glucan released in hydrothermal pretreatment of wheat straw were stronger inhibitors (∼100 times) of Cel7A than cellobiose. Furthermore, Ladisch and co-workers recently showed that in addition to starch and pectin, lignin derived phenols generated during biomass pretreatment can also deactivate enzymes (Kim et al., 2011; Ximenes et al., 2011). Consistent with our previous findings, their study also showed the strong inhibitory nature of xylooligomers to cellulase.

To reduce inhibition by plant derived inhibitors and increase sugars yields, cellulase preparations can be supplemented with high doses of accessory enzymes such as xylanase, pectinase, and β-glucosidase, or additives such as bovine serum albumin (BSA; Kumar and Wyman, 2009a,d; Yang and Wyman, 2006). However, studies are still limited on the effect of other plants derived compounds on cellulase activity. Herein, we report on our investigation of the effects of the non-xylan containing hemicellulose components heteromannans and their enzymatically prepared hydrolyzates (referred to as oligomers from here on) on cellulase activity.

Mannan polysaccharides are found in most lignocellulosic biomass feedstocks including softwoods, legumes, hardwoods, agricultural residues, dried distillers grain with solubles (DDGS), palm residues, and coffee residue waste, and yeast cell walls (Choi et al., 2012; Jørgensen et al., 2010; Kumar et al., 2010; Tucker et al., 2004). As shown in Figure 1, the mannan backbone is made up of β-1,4-linked D-mannose with almost no branching; glucomannan is a straight chain polymer of D-mannose and D-glucose randomly distributed units with a typical mass ratio of 1.6:1; and galactoglucomannan is basically a glucomannan with α-1,6 linked D-galactose side unit attached on the mannose residue. In galactoglucomannans, mannose can also be substituted with acetate at *0*-2 and *0*-3 positions (Shallom and Shoham, 2003).

Although a few studies reported the synergistic effects of supplementing endo-mannanase with cellulase on cellulose conversion (Banerjee et al. 2010a,b; Beukes et al., 2008; Sinner et al., 1979; Varnai et al., 2011), cellulase inhibition by mannan polysaccharides and their oligomers has never been reported. In this study, use of pure Avicel cellulose and commercial heteromannans revealed that heteromannans were highly inhibitory to cellulase activity and inhibition increased substantially with mannan substitution. We also found that mannan polysaccharides were stronger inhibitors than their respective oligomers and that even a high cellulase loading of 60 mg protein/g glucan and cellulase supplementation with commercial accessory enzymes (xylanase, pectinase, and β-glucosidase) was not effective in relieving inhibition for highly substituted mannan polysaccharides and their oligomers. However, further experiments showed that inhibition can be completely relieved by supplementing cellulase with purified accessory enzymes, suggesting that cellulase enzyme preparations would benefit substantially by including appropriate levels of such enzyme activities.

Materials and Methods

Substrates and Reagents

Avicel® PH 101 cellulose (>98% purity, Lot No. BCBD6923V, Fluka), beechwood xylan (BWX, >70% purity, Lot No. BCBS8393V), locust bean gum galactomannan (LBG-GalM, mannose to galactose weight ratio ∼4:1, Lot No. 120M0032V), and guar gum galactomannan (GG-GalM, mannose to galactose weight ratio ∼2:1, Lot No. 041M0058V) were purchased from Sigma–Aldrich (St. Louis, MO). Fenugreek gum galactomannan (FGG-GalM, mannose to galactose weight ratio ∼1:1, Sample No. 31474) and Tara gum galactomannan (TG-GalM, mannose to galactose weight ratio ∼3:1, Sample No. 31474) samples were kindly provided by Gum Technology Co., Tucson, AZ. Konjac roots derived glucomannan powder, a product of Konjac Foods, Sunnyvale, CA, and arabinogalactan, a product of Premier Research Labs, Austin TX (Lot No. 121011-2224), were purchased through Amazon.com. Ivory mannan (>98% purity, Lot No.10601b), high viscosity GG-GalM (Viscosity 17 dL/g; mannose to galactose ratio ∼1.63:1, Lot No. 100301a), medium viscosity GG-GalM (Viscosity 8 dL/g;

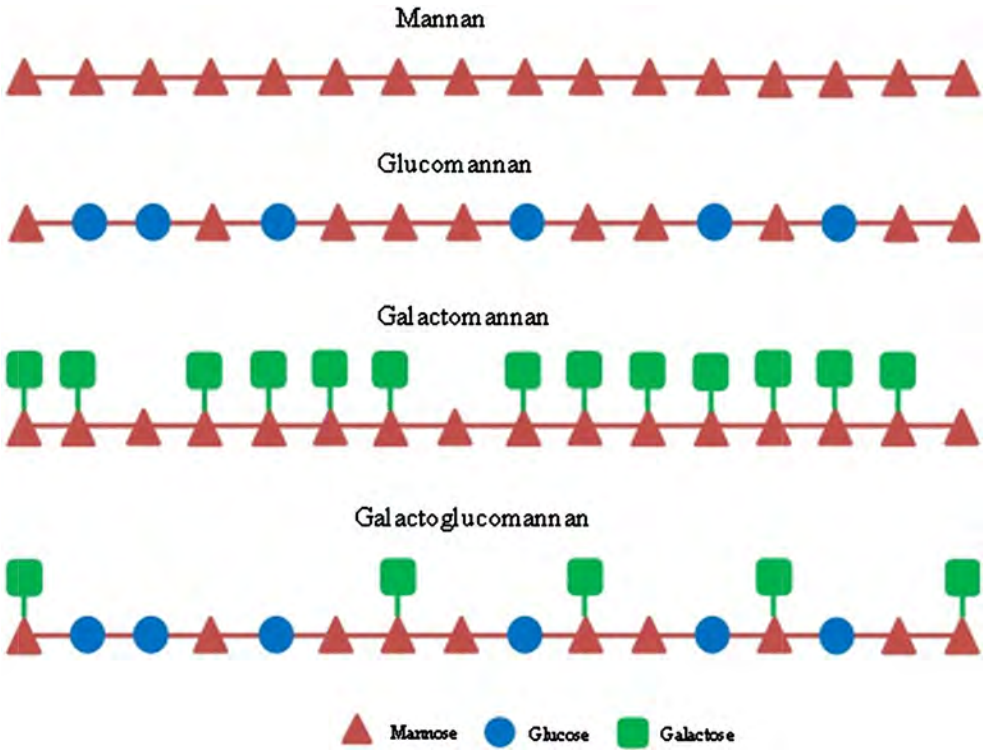

Figure 1. Schematic representation of mannan polysaccharides.

mannose to galactose ratio ~1.63:1, Lot No. 006079), and galactose depleted GG-GalM (mannose to galactose ratio ~3.76:1, Lot No. 105029, Viscosity 11 dL/g) were purchased from Megazyme International (Wicklow, Ireland). Sulfuric acid (72 wt %; Ricca) and other reagent grade chemicals were purchased from either Fisher Scientific (Pittsburgh, PA) or Sigma–Aldrich (St. Louis, MO).

Enzymes

Accellerase® 1500 cellulase (Protein content 82 ± 5 mg/mL), Multifect® xylanase (Protein content 42 ± 5 mg/mL), and Multifect® pectinase (Protein content 82 ± 7 mg/mL) were generously provided by DuPont™ Industrial Biosciences (formerly Genencor International), Palo Alto, CA. β-glucosidase Novozyme® 188, protein content 140 ± 10 mg/mL, a product of Novozymes, Inc., was purchased from Sigma–Aldrich. Enzymes protein contents were determined by applying the standard BCA protein assay (Smith et al., 1985). Purified *Aspergillus* niger endo-1, 4 β-mannanase (Protein content 11.8 mg/mL, Lot No. 120601a), Cellulomonas fimi β-mannosidase (Protein content 6.0 mg/mL, Lot No. 80501b), and Guar seed derived α-galactosidase (Protein content 6.15 mg/mL, Lot No. 70405b) were purchased from Megazyme International (Wicklow, Ireland). Substrate specificities of these purified enzyme components can be found elsewhere (Shallom and Shoham, 2003).

Preparation of Hemicellulose Oligomers

Oligomers of insoluble ivory mannan, water soluble konjac glucomannan (GluM), LBG-GalM, GG-GalM, TG-GalM, FGG-GalM, and arabinogalactan were prepared by 120 h enzymatic hydrolysis of a 5 wt% solids loading of the appropriate materials with a cellulase protein loading of ~30 mg/g dry solids in a 50 mM sodium citrate buffer containing 1 g/L sodium azide to prevent microbial growth followed by enzyme deactivation at 121°C for 20 min. Oligomers of beechwood xylan were prepared by hydrothermal pretreatment of 10 wt% beechwood xylan solids at 200°C for 15 min (Lloyd and Wyman, 2003). To separate unhydrolyzed solids from the liquid, the oligomer slurry was repeatedly centrifuged in a Beckman floor centrifuge (Model No. J2-21, Beckman Coulter, Inc., Brea, CA) at 10,000 rpm for 15 min followed by collection of virtually solids free supernatant. Then, the standard NREL method was applied to determine the monomeric equivalent concentration of dissolved sugar oligomers by post-hydrolysis in 4 wt% sulfuric acid at 121°C for 1 h followed by taking the difference in sugar concentrations before and after post-hydrolysis with adjustments made for any sugar loss in post-

hydrolysis by the appropriate sugar recovery standards (SRS) (Sluiter et al., 2008).

Composition Analysis

Substrates compositions were determined by the NREL standard method (Sluiter et al., 2008). In brief, primary hydrolysis was performed in triplicate in glass test tubes containing ~300 mg samples dry basis (<10 wt% moisture content) in 3 mL of 72 wt% sulfuric acid at 30 ± 1°C for 1 h followed by secondary hydrolysis in Wheaton serum bottles (Cat No. 06–06 J, Fisher Scientific, Pittsburgh, PA) in 4 wt% sulfuric acid at 121°C for 1 h. The appropriate SRS were run along in the secondary hydrolysis to provide a correction for sugar losses. National Institute of Standards and Technology (NIST) wheat straw (RM 8493) or an Eastern cottonwood (RM 8492) standard of known composition was also run to validate the accuracy of the method.

Enzymatic Hydrolysis

Following the NREL standard protocol, enzymatic hydrolysis of Avicel cellulose alone (control) and Avicel mixed with the different hemicelluloses added over a range of concentrations to 10 g/L glucan before enzymes addition was carried out in 125 mL Erlenmeyer flasks (Fisher Scientific) to which was also added 50 mM sodium citrate buffer (pH 5.0; 50°C, 150 rpm, total reaction weight 50 g) along with an Accellerase® 1500 cellulase protein loading of 15 mg/g glucan, unless noted otherwise (Selig et al., 2008). Sodium azide at a concentration of 1 g/L was also added to the hydrolysis slurry to prevent microbial growth. These reactions were carried out for 120 h with Multitron shakers (Model AJ125; Infors-HT, Laurel, MD) used to keep the materials mixed. GluM alone was also hydrolyzed in parallel at the same conditions to determine glucose released from GluM. To determine the effect of accessory enzymes on cellulose hydrolysis, Accellerase® 1500 cellulase at 15 mg protein/g glucan was supplemented with xylanase at 10 mg protein/g glucan, pectinase at 10 mg/g glucan, and β-glucosidase at 5 mg/g glucan. To determine the amount of sugars released and hydrolysis progress, about 1 mL of homogenous sample was withdrawn into a 2 mL micro-centrifuge tube (Catalog No. 05-402-95, Fisher Scientific) at various times over the 120 h hydrolysis followed by addition of about 15 μL of 10 wt% sulfuric acid and then centrifuged at 14, 6,000 rpm for 5 min in an Eppendorf centrifuge (Model No. 5424, Fisher Scientific). Sulfuric acid was added to stop hydrolysis, avoid the acid negative peak on HPLC for the Aminex® HPX 87-H column, and smooth integration of chromatograms. About 475 μL of solids free supernatant was then transferred into a 500 μL polypropylene snap ring vial (Vendor No. 98842; Grace Davision, Deerfield, IL) and run on HPLC for sugars analysis.

Hydrolysis experiments to determine the effect of supplementation of cellulase with purified accessory enzymes were run in 2 mL micro-centrifuge tubes (Catalog No. 05-402-95, Fisher Scientific) with a total reaction weight of 1.5 g. Avicel cellulose at 10 g/L glucan alone and mixed with GG-GalM at 2.5 g/L were hydrolyzed at 50°C for 24 h with Accellerase® 1500 cellulase at a protein loading of 15 mg/glucan. Cellulase (C) was supplemented with 10 mg protein/g glucan of any one of the following: endo-mannanase (E-M), β-mannosidase (Beta-M), α-galactosidase (Alfa-G), or their combinations (E-M plus Beta-M, Beta-M plus Alfa-G, E-M plus Alfa-G, and E-M plus Beta-M and Alfa-G). Other hydrolysis conditions were as above except samples were hydrolyzed by mounting on an end-over-end rugged rotator (Glass-Col, LLC, Terre Haute, IN) controlled by a DC motor at ~20 rpm. Cellulose conversion was calculated as:

$$\text{Cellulose conversion}\,(\%) = 100^{*}[0.90^{*}(\text{Glucose}\,(\text{g/l}) + 1.053^{*}\text{Cellobiose}\,(\text{g/l}))]/\text{Initial cellulose}\,(\text{g/l})$$

where 0.90 and 1.053 are the mass conversion factors based on the stoichiometry for conversion of glucose to cellulose and cellobiose to glucose, respectively.

The reduction in cellulose conversion due to added components was calculated as:

$$\%\,\text{Relative drop} = 100 \times \left[\frac{\%\,\text{Cellulose conversion for Avicel control} - \%\,\text{Cellulose conversion for Avicel mixed with heteromannans, their oligomers, or other inhibitors}}{\%\,\text{Cellulose conversion for Avicel control}}\right]$$

Analysis

Liquid samples from composition analysis and enzymatic hydrolysis were analyzed for sugars on a Waters Alliance HPLC (Model e2695) equipped with an auto sampler (Waters 2695) and a 2414 refractive index (RI; Waters Co., Milford, MA) detector. A Bio-Rad Aminex® HPX-87H (Polystyrene-divinylbenzene sulfonic acid resin packing; 300 mm × 7.8 mm; Catalog No.125–140) column along with a micro-guard cation cartridge (Catalog No.125–129; 30 mm × 4.6 mm; Bio-Rad Laboratories, Hercules, CA) were used. The column was heated to 65°C, with 5 mM sulfuric acid at a flow rate of 0.6 mL/min as the carrier solvent. Since galactose, mannose, and xylose co-elute together on a Bio-Rad Aminex® HPX-87H column, concentration of these individual sugars was determined by running the samples on a Bio-Rad Aminex® HPX-87P as well. Samples collected for compositional analysis were neutralized first to about pH 5.0 with $CaCO_3$.The column was heated to 80°C, with double deionized water at a flow rate of 0.6 mL/min as the carrier solvent. The chromatograms were integrated, and data was imported to Microsoft Excel files using Empower 2 software (Waters Co., Milford, MA).

Results

Compositional analyses of commercial hemicelluloses showed that all of them contained negligible amounts of

Table I. Composition of heteromannans used in the study.

Substrate	Avg. wt%, dry basis						Total mass, %	Man:Gal
	Glu	Xyl	Gal	Ara	Man	Lignin		
Mannan	ND				>98	ND	100.0	NA
Glucomannan	41.2	0.0	0.0	0.0	59.9	0.1	101.0	NA
Locust bean gum galactomannan	2.4	0.5	19.1	0.0	68.4	1.8	92.2	3.6:1
Tara gum galactomannan	1.70	0.0	21.2	0.0	67.6	0.8	91.3	3.2:1
Guar gum galactomannan	4.7	1.4	31.0	0.0	54.0	1.5	92.5	1.8:1
Fenugreek gum galactomannan	1.96	0.0	40.8	0.0	47.1	0.2	90.1	1.2:1

Glu, glucan; Xyl, xylan; Gal, galactan; Ara, Arabinan; Man, mannan; NA, not applicable; ND, not determined.

lignin, and the mannose to galactose weight ratio in the galactomannans varied from about 3.6:1 in LBG-GalM to 1.2:1 in FGG-GalM (Table I). Glucomannan contained about 59 wt% glucan and 41 wt% mannan (glucan to mannan ratio: 1.44), whereas BWX was 71% pure. Mannan and Megazyme GG-GalMs, as per the manufacturer's specifications, were >98% pure, and their compositions were as specified by the manufacturer.

Effect of Heteromannan Types on Cellulase Activity

Enzymatic hydrolysis of Avicel cellulose was performed at a 10 g/L glucan loading with and without (control) various hemicellulose heteromannans including mannan, GluM, LBG-GalM, TG-GalM, GG-GalM, and FGG-GalM, and mannose added to the hydrolysis slurry before cellulase addition. The heteromannan loading was 2.5 g/L (heteromannan to cellulose wt ratio = 0.25:1) consistent with its ratio to cellulose in typical biomass, and mannose was loaded at 10 g/L, as a lower loading of 2.5 g/L had no inhibitory effect on cellulase activity. Figure 2a illustrates the effects of various heteromannan types and mannose monomer on cellulose initial hydrolysis rates and final 120 h conversions at a cellulase loading of 15 mg protein/g glucan. As summarized in Table II, Avicel cellulose conversions at 4 h and 120 h were 24.3 and ~91%, respectively, but adding heteromannans or mannose to Avicel dropped conversions significantly. At the extreme, highly substituted heteromannan, that is, FGG-GalM, resulted in the lowest Avicel conversion of only 11.5% after 120 h followed by a 120 h cellulose conversion of just 29.3% when GG-GalM was added to Avicel. On the other hand, 10 g/L of mannose was the least inhibitory to Avicel conversion, with a 4.6% relative drop in 120 h conversion. Both mannan and glucomannan had similar relative drops of ~22% at 120 h, but GluM more strongly inhibited initial (4 h) rates than mannan (76.5% relative drop vs. 43.6% for mannan). Cellulase inhibition dramatically increased with mannose backbone substitution of the galactose residue, with the relative drop in 4 h Avicel hydrolysis rates being somewhat greater when mixed with less substituted mannan (79.4% drop by LBG-GalM and 79.8% by TG-GalM) than when mixed with highly substituted mannan (66.3% for GG-GalM and 73.3% by FGG-GalM). However, Table II shows that the relative drop in cellulose conversion for Avicel mixed with

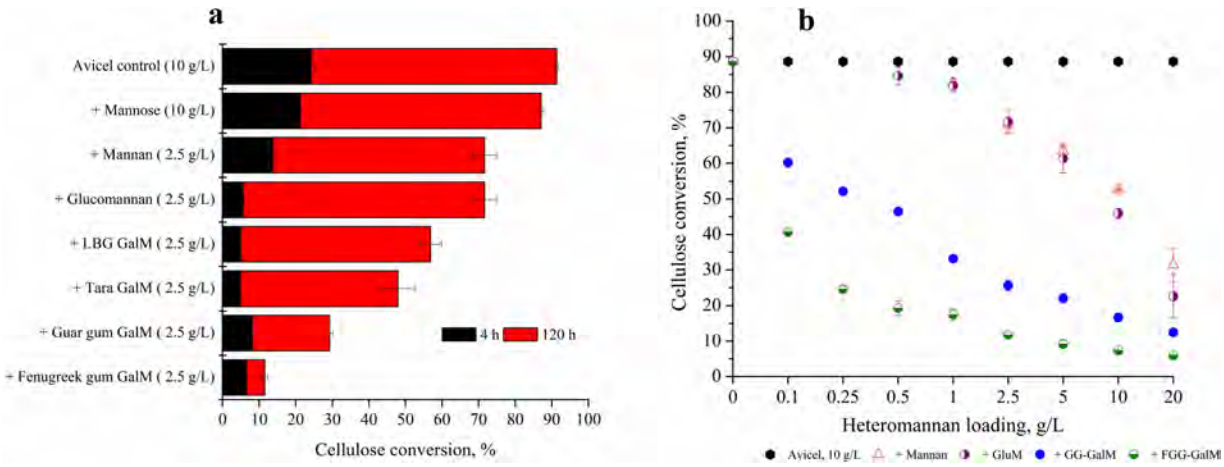

Figure 2. **a**: Effect of 2.5 g/L of various heteromannans and 10 g/L of mannose on initial 4 h and final 120 h conversions of Avicel cellulose (10 g/L) at an Accellearse®1500 cellulase protein loading of 15 mg/g glucan. **b**: Effect of concentrations of the heteromannans mannan, glucomannan (GluM), guar gum (GG) galactomannan (GalM), and fenugreek gum (FGG) GalM (g/L) on 120 h Avicel cellulose conversion at an Accellearse®1500 cellulase protein loading of 15 mg/g glucan. Note: Enzymatic hydrolysis was performed in a 50 mM sodium citrate buffer (pH 5.0) at 50°C and 150 rpm for 120 h. Heteromannans were added to the hydrolysis slurry before cellulase addition. LBG, locust bean gum.

Table II. Summary of the effect of various heteromannans and mannose on Avicel cellulose conversion and percent relative drop in conversion at a cellulase protein loading of 15 mg/g glucan.

	Cellulose conversion, %					% Relative drop in conversion over control				
Substrate	4 h	24 h	72 h	96 h	120 h	4 h	24 h	72 h	96 h	120 h
Avicel cellulose alone (10 g/L; control)	24.3	58.1	81.7	87.2	91.3			NA		
+ Mannose (10 g/L)	21.2	46.6	75.5	81.5	87.1	12.8	15.3	7.6	6.5	4.6
+ Mannan (2.5 g/L)	13.7	41.0	63.0	68.2	71.6	43.6	25.5	22.9	21.8	21.6
+ GluM (2.5 g/L)	5.7	34.3	59.2	66.6	71.6	76.5	37.6	27.5	23.6	21.6
+ LBG-GalM (2.5 g/L)	5.0	26.5	48.1	52.9	56.9	79.4	51.8	41.1	39.3	37.7
+ TG-GalM (2.5 g/L)	4.9	26.8	43.6	46.3	47.9	79.8	51.3	46.6	46.9	47.5
+ GG-GalM (2.5 g/L)	8.2	16.8	25.1	27.2	29.3	66.3	69.5	69.3	68.8	67.9
+ FGG-GalM (2.5 g/L)	6.5	9.5	10.8	11.2	11.5	73.3	82.7	86.8	87.2	87.4

NA, not applicable; GluM, glucomannan; LBG, locust bean gum; TG, tara gum; GG, guar gum; FGG, fenugreek gum; GalM, galactomannan.

LBG-GalM or TG-GalM decreased with hydrolysis, whereas it remained the same or increased for Avicel mixed with GG-GalM or FGG-GalM with the relative drop in 120 h conversion increasing in the following order: LBG-GalM (~37.8%) < TG-GalM (~47.5%) < GG-GalM (~68.0%) < FGG-GalM (~87.4%). The 120 h hemicellulose mannan conversion (data not shown; accounting only monomers) for mixtures with mannan and GluM at a cellulase loading of 15 mg protein/g glucan was about 11% and 24.4%, respectively, whereas mannan plus galactan conversions for mixtures with LBG-GalM, TG-GalM, GG-GalM, and FGG-GalM were 11.8%, 10.7%, 7.4%, and 4.2%, respectively (data not shown).

Effect of Heteromannan Concentration on Cellulase Activity

Initial screening of heteromannan types above showed that cellulase was strongly inhibited by heteromannans, even at a moderate loading of 2.5 g/L. We further explored the effect of heteromannan concentration on cellulase activity for cellulose hydrolysis at a constant glucan loading of 10 g/L and by varying the heteromannan concentration from 0.1 to 20 g/L. Figure 2b and Table S1 (Supporting Information) summarize the effects of heteromannan concentrations ranging from 0.1 to 20 g/L on the relative drops in cellulose conversion. Although concentrations of mannan and GluM below 2.5 g/L were less inhibitory (<8% drop in conversion), inhibition rapidly increased with higher loadings, with the result that the drop in 120 h cellulose conversion was 64.4% and 74.9% at mannan and GluM concentrations of 20 g/L, respectively. On the other hand, the presence of GG or FGG-GalM drastically increased the relative drop in cellulose conversion and resulted in much greater effects on hydrolysis rates than unsubstituted mannan or GluM at similar concentrations. For example, at an enzyme loading of 15 mg cellulase protein/g glucan, a 1 g/L concentration of GG and FGG-GalM decreased Avicel cellulose conversion by >60% and >80%, respectively, while mannan and GluM had an insignificant drop of 6–8% at the same concentration. Furthermore, surprisingly, even a concentration of 0.1 g/L (i.e., cellulose to GalM wt ratio = 100:1; GalM to cellulase mass ratio = 0.67:1) of GG-GalM (a highly substituted mannan with mannose to galactose weight ratio of 1.6:1) and FGG-GalM (a highly substituted mannan with mannose to galactose weight ratio of 1.2:1) dropped cellulose conversion by 32 and 54.1%, respectively. Figure 2b and Table S1 (Supporting Information) also show that at all concentrations, FGG-GalM inhibited cellulase more than GG-GalM.

Comparison of Cellulase Inhibition by Hemicellulose Polymers Versus Oligomers

To compare the effects of unhydrolyzed mannan polysaccharides and their respective enzymatically prepared oligomers (see Materials and Methods) on cellulase, enzymatic hydrolysis of Avicel cellulose alone and mixed with 2.5 and 10 g/L of heteromannan polymers and their respective enzymatically derived oligomers was performed at a cellulase protein loading of 15 mg/g glucan for 120 h. For comparison, hydrolysis was also performed on Avicel cellulose mixed with BWX and its hydrothermally derived XOs. Data shown in Figure 3a and b for hemicellulose polymers and their oligomers at concentrations of 2.5 and 10 g/L, respectively, illustrate that unhydrolyzed polysaccharides in general were more inhibitory than their respective oligomers except BWX. Cellulase inhibition by 2.5 g/L of mannan, GluM, LBG-GalM, or TG-GalM oligomers was marginal (<12% relative drop), while increasing the concentration of these same species to 10 g/L almost doubled the relative drop, with the GluM oligomers having the greatest effect of an ~28% relative drop. However, although only 2.5 g/L was highly inhibitory for GG and FGG-GalM oligomers, with relative drops of 45.3% and 81%, respectively, their effect on cellulose conversion increased only marginally at 10 g/L. It is important to note here that the oligomers used in this study were prepared by a prolonged enzymatic hydrolysis with a relatively high loading of cellulase (30 mg protein/g glucan). However, the structure of oligomers generated in enzymatic hydrolysis at low enzyme loadings and in pretreatment (Kang et al., 2012; Kont et al., 2013; Kumar et al., 2010) might be different in terms of their composition, backbone substitution, and chain length etc., and may prove to be more inhibitory than the inhibition

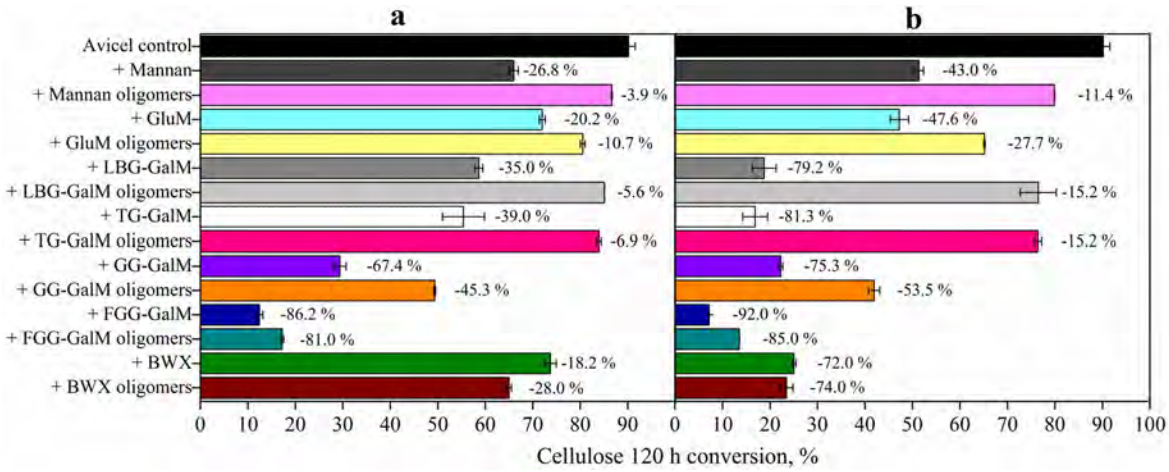

Figure 3. Effect of hemicellulose polymers and their oligomers at concentrations of (**a**) 2.5 g/L and (**b**) 10 g/L on 120 h Avicel cellulose conversion for an Accellerase® 1500 cellulase loading of 15 mg protein/g glucan. Note: Number noted on the graph is the percent relative drop in cellulose conversion by the respective hemicellulose and their oligomers compared to the control. Cellulose enzymatic hydrolysis was performed at a 10 g/L glucan loading in a 50 mM sodium citrate buffer (pH 5.0) at 50°C and 150 rpm. Polymers or their respective oligomers were added to the hydrolysis slurry before cellulase addition. Heteromannan oligomers were prepared enzymatically, whereas beechwood xylan (BWX) oligomers were prepared by hydrothermal pretreatment at 200°C for 15 min. GluM, glucomannan; GalM, galactomannan; LBG, locust bean gum; TG, tara gum; FGG, fenugreek gum; GG, guar gum.

reported here for extensively hydrolyzed mannan polysaccharides. However, further work is in progress to confirm this hypothesis. As also shown in Figure 3, 2.5 g/L of BWX was less inhibitory, with only a 18.2% relative drop in conversion, than any of the heteromannans. However, 10 g/L of BWX resulted in a 72.2% relative drop in cellulose conversion while unsubstituted mannan and GluM reduced it by 43% and 47.6%, respectively. Furthermore, 10 g/L of XOs were more inhibitory (74% relative drop in 120 h conversion) than the oligomers of heteromannans at equal concentration (with less than 54% relative drop), except FGG-GalM oligomers, which produced a 85% relative drop in 120 h cellulose conversion.

Effect of Cellulase Loading on Cellulase Inhibition by GG-GalM

To evaluate whether high cellulase loadings can overcome the drastic inhibition by GalM, Avicel cellulose alone and mixed with GG-GalM at concentrations from 1 to 20 g/L was enzymatically hydrolyzed at cellulase protein loadings of 5, 10, 15, 30, and 60 mg/g glucan, as shown in Figure 4 for GG-GalM concentrations of 1 and 10 g/L and in Figure S1 (Supporting Information) for the rest of the conditions. Figure 4 shows that inhibition by GalM present in the hydrolysis slurry at 1 g/L can be removed to some extent by increasing the cellulase loading; however, about a 24% relative drop in 120 h cellulose conversion still was evident even at the very high cellulase loading of 60 mg protein/g glucan. Furthermore, it appears that the hydrolysis slurry with GG-GalM present at 1 g/L required about six times as much cellulase protein was needed to achieve conversions similar to those for the control. For instance, loadings of 30 and 60 mg cellulase protein/g glucan were almost able to give the same 120 h conversions of 10 g/L of cellulose mixed with 1 g/L of GG-GalM to those for the control at 5 and 10 mg protein/g glucan, respectively. Figure 4 also shows the effect of cellulase loading on yields of glucose from Avicel in the presence of 10 g/L of GG-GalM. In contrast to hydrolysis results at lower GG-GalM concentrations, increasing cellulase loadings did not have much effect on the relative drop in cellulose conversion. For example, at a 5 mg/g glucan

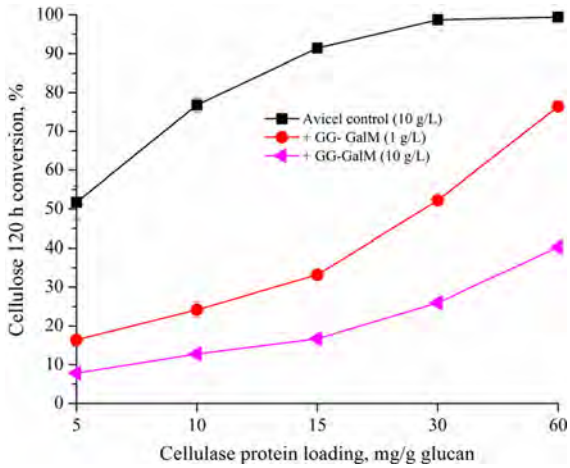

Figure 4. Cellulose conversion vs. Accellerase® 1500 cellulase protein loadings for Avicel cellulose alone (control; 10 g/L) and Avicel cellulose mixed with guar gum (GG) galactomannan (GalM) at 1.0 and 10 g/L. Note- Enzymatic hydrolysis was performed in a 50 mM sodium citrate buffer (pH 5.0) at 50°C and 150 rpm for 120 h. GG-GalM was added to the hydrolysis slurry before cellulase addition.

cellulase protein loading, 120 h cellulose conversion was less than 10%, about a 85% relative drop from the 54% yield for the control, but increasing the cellulase loading to 60 mg/g glucan increased the yield to about 38%, about a 60% relative drop compared to the virtually complete hydrolysis for the control at the higher loading. Furthermore, in the presence of 10 g/L of GG-GalM, even the highest cellulase loading of 60 mg/g glucan was not able to match cellulose conversion to results with the control at a cellulase loading of only 5 mg/g glucan. Figure S1 (Supporting Information) shows that 20 g/L of GG-GalM resulted in a relative drop in cellulose conversion of 90% at 5 mg and ~70% at 60 mg protein/g glucan loading.

Effect of Accessory Enzymes on Cellulase Inhibition by Heteromannans

To reduce inhibition and enhance cellulose accessibility in pretreated biomass to cellulase, cellulase is often supplemented with accessory enzymes (Berlin et al., 2007; Penttilä et al., 2013; Varnai et al., 2011; Zhang et al., 2011). Therefore, to determine whether the strong inhibition of cellulase by heteromannans could be alleviated by supplementation of cellulase with accessory enzymes, hydrolysis of cellulose alone (10 g/L) and mixed with mannan, GluM, LBG-GalM, or GG-GalM (at 2.5 g/L concentration) was performed with cellulase alone (15 mg protein/g glucan) and cellulase supplemented with Multifect® xylanase (Mxy; 10 mg protein/g glucan), Multifect® pectinase (Mpe; 10 mg protein/g glucan), and β-glucosidase (Beta-g; 5 mg protein/g glucan). Here it is important to note that the aim of this set of experiments was not to optimize the mass ratios of these various enzymes but rather to determine whether typical loadings of these commercial enzymes would alleviate cellulase inhibition by heteromannans. For hydrolysis of Avicel mixed with mannan and GluM, Figure 5a shows that supplementation with accessory enzymes alleviated cellulose inhibition virtually completely, and the 120 h conversions for both Avicel mixed with mannan and GluM were almost equal to the control. For the LBG-GalM data in Figure 5b, although supplementation of cellulase with commercial enzymes, even at such high loadings, was not very effective in removing inhibition in the initial phase of hydrolysis (>50% relative drop after 4 h), it virtually eliminated cellulase inhibition after 120 h (<8% relative drop compared to the control), suggesting that accessory enzymes hydrolyzed LBG-GalM sufficiently so that it was no longer inhibitory to cellulase. On the other hand, for hydrolysis of Avicel mixed with 2.5 g/L of GG-GalM (a highly substituted mannan), Figure 5c shows that supplementation of cellulase with accessory enzymes only increased 120 h cellulose conversion marginally from 24.6% to 38.1%. Furthermore, applying twice the loading of accessory enzymes used in Figure 5a, that is, 20 mg protein/g glucan of Mxy and Mpe, each, and 10 mg protein/g glucan of Beta-g to 15 mg protein/g glucan of cellulase only increased cellulose conversion to 47.8% (data not shown). Although cellulase inhibition by GG-GalM oligomers was comparatively less than by unhydrolyzed GG-GalM polymer (shown in Fig. 3), supplementation of cellulase with accessory enzymes did not improve yields much for even hydrolysis of Avicel cellulose mixed with GG-GalM oligomers (Fig. S2). This data indicate that for highly substituted mannans, merely supplementing cellulase with commercial accessory enzymes does not remove inhibition if the enzyme preparations used lack the activities required to hydrolyze highly substituted mannan.

Further, experiments were carried out with purified commercial enzymes to investigate their effect on cellulase inhibition by GG-GalM. Accellerase®1500 cellulase at 15 mg protein/g glucan was supplemented with E-M at 10 mg protein/g glucan, Beta-M at 10 mg protein/g glucan, Alfa-G at 10 mg protein/g glucan, or the following combinations: E-M + Beta-M, each at 10 mg protein/g glucan; Beta-M + Alfa-G, each at 10 mg protein/g glucan; E-M + Alfa-G, each at 10 mg protein/g glucan, and E-M + Beta-M + Alfa-G, each at 10 mg protein/g glucan. Figure 5d shows the 24 h hydrolysis data for the effect of supplementation of cellulase with purified accessory enzymes on hydrolysis of Avicel cellulose at 10 g/L mixed with GG-GalM (2.5 g/L). The numbers shown on the graph are the percent relative drop in cellulose conversion. Consistent with previous sections, GG-GalM resulted in a 70.6% relative drop in cellulose conversion. Although supplementation with just E-M or Alfa-G removed inhibition to some extent, the percent relative drop in cellulose conversion was still >50%. However, when used together, E-M and Alfa-G removed cellulase inhibition almost completely and reduced the percent relative drop in cellulose conversion from 70.6% to only ~18%, strongly suggesting the importance of these two enzymes in overcoming cellulase inhibition by GG-GalM. Consistent with this, Clarke et al. (2000) also reported synergism between E-M and Alfa-G as the supplementation of Alfa-G with E-M enhanced the activity of the former in removing galactose from softwood pulp. However, supplementation of cellulase with Beta-M, a key enzyme to hydrolyze mannan oligomers, by itself or in combination with other enzymes, did not have much effect on cellulose conversion.

Discussion

Because enzymes are expensive and yields must be high to spread costs, realizing a high sugar yield at low enzyme loadings is vital to converting lignocellulosic biomass to fuels and chemicals at costs competitive with fossil fuels. Prior to biological conversion, most forms of lignocellulosic biomass must be pretreated to realize high yields by enhancing its accessibility to enzymes and microorganisms (Karimi et al., 2013; Kumar et al., 2009; Yang and Wyman, 2008). However, as discussed elsewhere in detail (Kumar and Wyman, 2010, 2013), lignocellulosic biomass hydrolysis with cell free enzymes can be considered to be controlled by two main factors: (1) biomass accessibility to enzymes; and (2) enzyme effectiveness. Biomass accessibility can further be categorized according to macro and micro-accessibility (Kumar and Wyman, 2013). Although most thermo-

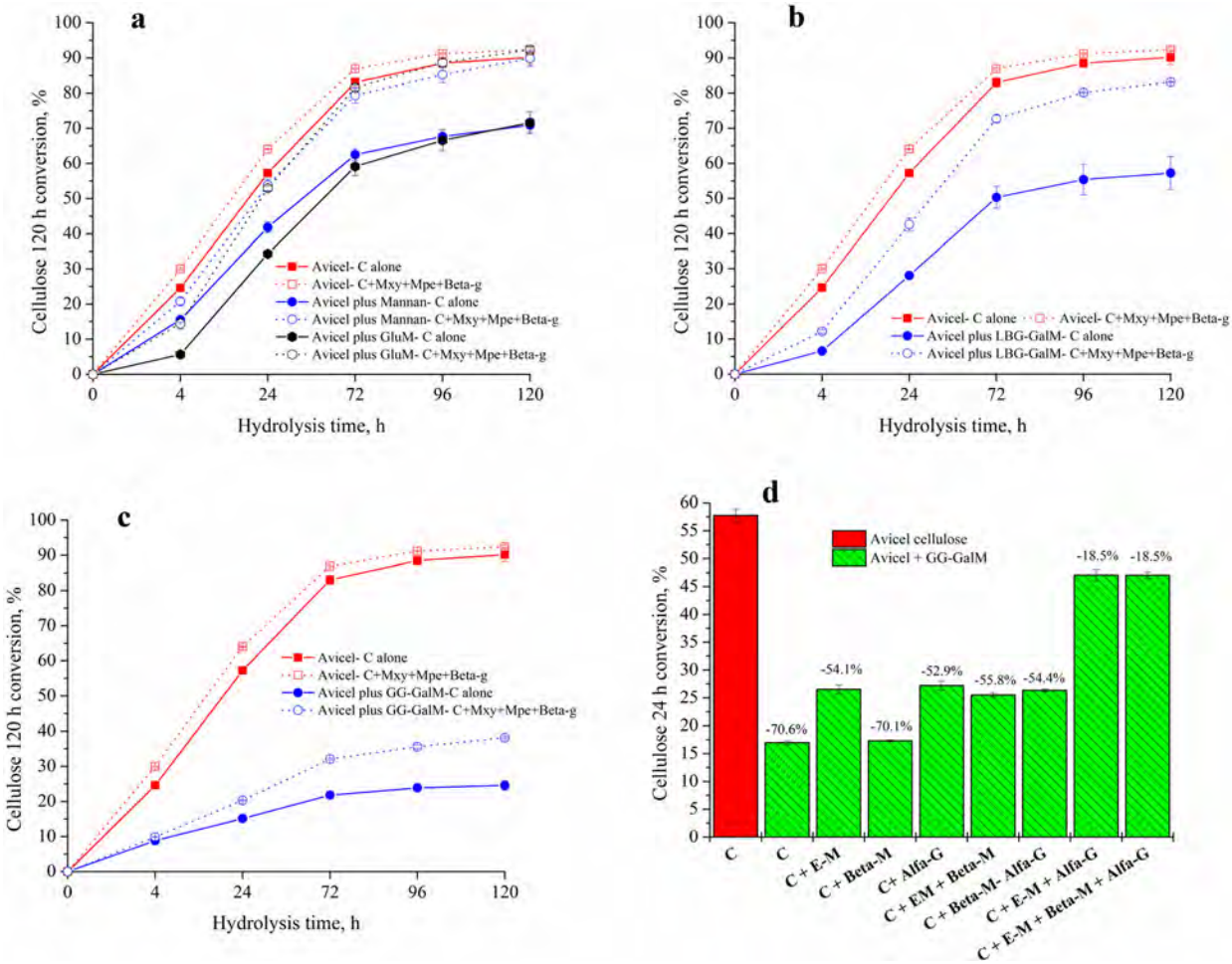

Figure 5. Effect of supplementation of cellulase with accessory enzymes on cellulose conversion for 10 g/L of Avicel cellulose alone (control) and Avicel cellulose mixed with 2.5 g/L of (**a**) mannan and glucomannan (GluM), (**b**) locust bean gum (LBG) galactomannan (GalM), and (**c**) guar gum (GG)-GalM. (**d**) Effect of supplementation of cellulase with purified accessory enzymes on cellulose 24 h conversion for Avicel cellulose alone (control; 10 g/L) and Avicel cellulose mixed with 2.5 g/L GG-GalM. Note: In Figure 4a–c, Accellerase® 1500 cellulase (C) at a loading of 15 mg protein/g glucan was supplemented with Multifect® xylanase (Mxy) at 10 mg protein/g glucan, Multifect® pectinase (Mpe) at 10 mg protein/g glucan, and Novozyme® 188 β-glucosidase (Beta-g) at 5 mg protein/g glucan. In Figure 5d, Accellerase® cellulase (C) at a loading of 15 mg/g glucan was supplemented with purified endo-mannanase (E-M; 10 mg protein/g glucan), β-mannosidase (Beta-M; 10 mg protein/g glucan), α-galactosidase (Alfa-G; 10 mg protein/g glucan), or their combinations. Number noted on the graph 5d is the percent relative drop in cellulose conversion by the respective GalM compared to the control.

chemical pretreatments improve biomass macro-accessibility by removing/dislocating lignin and/or hemicellulose, micro-accessibility of carbohydrates and enzyme effectiveness in the hydrolysis system are still big challenges to realizing high sugar yields at low protein loadings (Kumar and Wyman, 2013).

Glucose and cellobiose, end-products of cellulose hydrolysis, lower enzyme effectiveness by inhibiting enzymes in a competitive/non-competitive manner (Andrić et al., 2010). However, we recently showed that hemicellulose xylan and its oligomers released during hemicellulose breakdown are also strong inhibitors of cellulase action and retard its effectiveness even more than glucose and cellobiose, whose effects have been traditionally recognized (Kumar and Wyman, 2009b,c; Teugjas and Valjamae, 2013). Although heteromannans also comprise a significant fraction of the hemicellulose in biomass, possible inhibition of cellulase activity by these breakdown products has never been considered. In this study, we have shown that heteromannans at a concentration as low as 0.1 g/L can greatly affect cellulase effectiveness (Fig. 2b) and that effectiveness was further suffered when the mannan backbone was substituted with galactose residues. Since all the heteromannans used in this study did not contain a significant amount of lignin/pseudo-lignin (Table I), enzymes unproductive binding to lignin and/or pseudo-lignin (Kumar et al., 2013; Li et al., 2014) can be ruled out as the cause for the drop in cellulose conversion.

Although heteromannans, mainly in the form of glucomannan, only comprise ~5–7 wt% of the total mass of agricultural residues, energy crops, and hardwoods, the hydrolysis system can contain up to 10–15 g/L of GluM for enzymatic hydrolysis of the high solids (30–50 wt%)

concentrations required for commercial success, thus significantly reducing cellulase effectiveness (Fig. 2b and Table S1). For softwoods, on the other hand, heteromannans (~15–20 wt%) are in the form of galactoglucomannan (GGM) with average weight ratios of mannose, glucose, and galactose varying from 3:1:1 to 3:1:0.1 (Wyman et al., 2005). Accordingly, the hydrolysis system for softwoods can contain more than 40 g/L of mannan polysaccharides for enzymatic hydrolysis at high solids (Kang et al., 2012). Herein, by using commercial galactomannans (GalMs), we showed that GalMs even at an extremely low concentration of 0.1 g/L were highly inhibitory to cellulose conversion and reduced cellulase effectiveness up to 90%. In addition, when compared to other inhibitors, Figure S3 (Supporting Information) shows that GalMs were stronger inhibitors than cellobiose at 2.5 g/L, furfural at 2.5 g/L, and XOs/xylan (as shown in Fig. 3) at much lower or equal concentrations. However, to evaluate whether substituted mannan polysaccharides are stronger inhibitors than cellobiose and XOs, and establish inhibition mechanism, further research with purified enzymes is needed.

As shown in Figure 2a, LBG-GalM caused a greater drop in initial rates than GG-GalM, possibly due to higher molecular weight (MW) of the former resulting in a more viscous slurry that limited enzymes mobility. Therefore, to further investigate the effect of molecular weight on cellulase inhibition, hydrolysis was performed with Avicel mixed with a high viscosity (17 dL/g; Megazyme GG-GalM-1) and a medium viscosity (8 dL/g; Megazyme GG-GalM-2) guar gum purchased from Megazyme. Figure 6 shows that higher viscosity did not have a significant effect on either the initial rate or final conversion of Avicel cellulose. Further experiments were carried out to confirm the observations made in Figure 2a and b that cellulase inhibition increased with mannose backbone substitution with galactose. In this case, enzymatic hydrolysis was applied to Avicel alone and Avicel mixed with regular GG-GalM (Megazyme GG-GalM-1; mannose to galactose ratio 1.63:1) and galactose depleted GG-GalM (Megazyme GG-GalM-3; mannose to galactose ratio 3.76:1), and the results compared to those for hydrolysis of Avicel mixed with Sigma GG-GalM (mannose to galactose ratio 1.8:1) and Sigma LBG-GalM (mannose to galactose ratio 3.6:1). Figure 6 shows that the percent relative drop in cellulose conversion for galactose depleted GG-GalM was much lower than for regular GG-GalM and nearly equal to that for LBG-GalM that had a similar mannose to galactose ratio as Megazyme galactose depleted GalM. These results confirmed that galactose substitution in heteromannans increased cellulase inhibition.

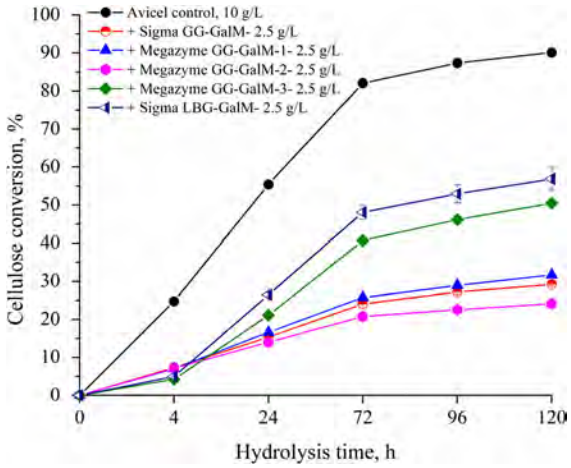

Figure 6. Effect of galactomannan (GalM) molecular weight and galactose substitution on cellulase effectiveness for Avicel cellulose (10 g/L) conversion. Note: Enzymatic hydrolysis on Avicel cellulose alone and Avicel cellulose mixed with 2.5 g/L of high viscosity guar gum (Megazyme GG-GalM-1; viscosity 17 dL/g; mannose to galactose ratio ~1.63:1), medium viscosity guar gum (Megazyme GG-GalM-2; viscosity 8 dL/g; mannose to galactose ratio ~1.63:1), and galactose depleted guar gum (Megazyme GG-GalM-3; viscosity 11 dL/g; mannose to galactose ratio ~3.76:1) from Megazyme, and guar gum (Sigma-GG-GalM; mannose to galactose ratio ~1.8:1) and locust bean gum (Sigma-LBG-GalM; mannose to galactose ratio ~3.6:1) GalM from Sigma was performed in 50 mM (pH 5.0) sodium citrate buffer at 50°C and 150 rpm at an Accellerase®1500 cellulase protein loading of 15 mg/g glucan.

Although further research is needed to pinpoint an inhibition mechanism, based on literature, it appears that mannan strongly adsorbs onto cellulose surface by forming hydrogen bonds with cellulose hydroxyl groups as a result of their similar structures (Hansson, 1970; Mishima et al., 1998; Newman and Hemmingson, 1998; Whitney et al., 1998). The resulting cellulose surface coverage with mannan may affect cellulase effectiveness and/or cellulose accessibility resulting in a dramatic drop in cellulose conversion. For example, Clayton and Phelps (1965) compared adsorption of xylan and glucomannan on α-cellulose wood fibers and reported that twice as much GluM was adsorbed as xylan. Hannuksela et al. showed that soluble O-acetyl galactoglucomannans isolated from pulp and guar gum galactomannan strongly adsorbed on kraft pulp, and deacetylation of GGMs enhanced their adsorption (Hannuksela et al., 2002; Hannuksela et al., 2003). Eronen et al. by applying quartz crystal microbalance with dissipation (QCM-D) quantified the amount and strength of hemicelluloses adsorption on nanofibrillar cellulose, and concluded that GGMs had a tighter association with cellulose fibrils than arabinoxylan, but xyloglucan had the strongest attachment (Eronen et al., 2011). In another recent study, Parikka et al. quantified the adsorption of spruce GGM on cellulose to be about 1 mg/m^2, and only 13% desorbed when rinsed with Milli-Q water, suggesting a strong association of GGMs with cellulose (Parikka et al., 2012). Furthermore, although the GluM and GalM used in this study were soluble, cellulase may also bind to insoluble mannan, resulting in reduced enzyme availability and effectiveness (Tenkanen et al., 1995). Cellulase inhibition was also seen to drastically increase with mannan substitution and was very high even at a miniscule concentration of 0.1 g/L (>30%, Fig. 2b, Table S1, and Fig. 6), suggesting that galactose substitution of the mannan backbone possibly resulted in greater and/or stronger adsorption onto cellulose. In Figure 7, lower inhibition by GG and FGG-GalM at higher cellulose to GalM weight ratios of 200 and 500, while keeping constant

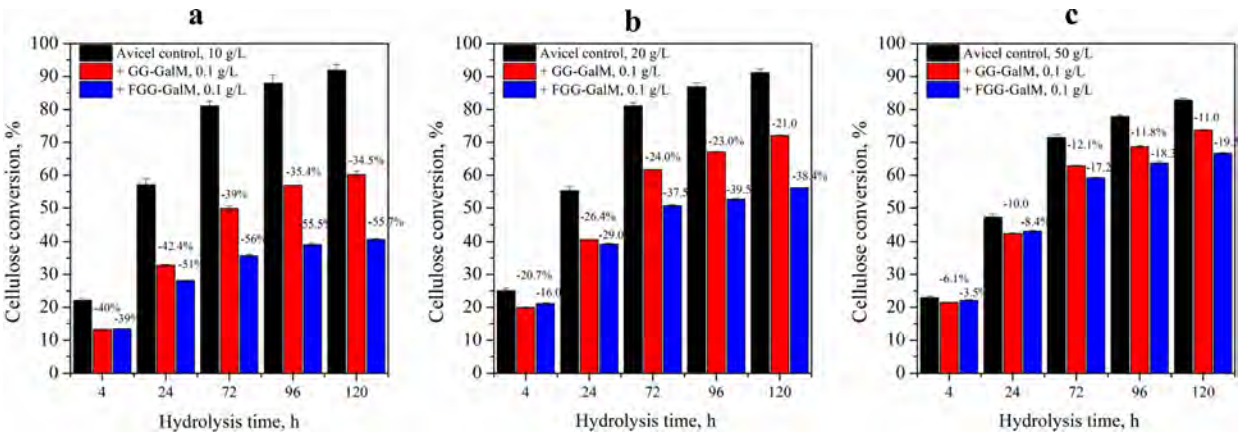

Figure 7. Effect of fixed guar gum (GG) or fenugreek gum (FGG) galactomannan (GalM) loading of 0.1 g/L on cellulose conversion at loading of (**a**) 10 g/L (cellulose to GalM wt ratio = 100:1), (**b**) 20 g/L (cellulose to GalM wt ratio = 200:1), and (**c**) 50 g/L (cellulose to GalM wt ratio = 500:1). Note: Enzymatic hydrolysis in all cases was performed in 50 mM sodium citrate buffer at 50°C and 150 rpm at Accellerase® 1500 cellulase loading of 15 mg/g glucan. Numbers on the graphs are % relative drop in cellulose conversion for Avicel mixed with GG-GalM or FGG-GalM over control.

cellulase protein loading of 15 mg/g glucan, that is, constant GalM to cellulase mass ratio, also indicated a stronger attachment of highly substituted GalM to the cellulose surface. In addition, the lower inhibition by GalMs oligomers than by their respective unhydrolyzed polymers except FGG-GalM also suggested stronger and higher adsorption of the polymer onto cellulose, which is consistent with findings by (Mishima et al., 1998). However, some studies pointed out that increased substitution of the mannan backbone reduced its adsorption onto cellulose (Hannuksela and Holmbom, 2003; Hannuksela et al., 2002; Newman and Hemmingson, 1998). Hannuksela and Holmbom (2003) suggested that due to galactose side groups, mannan will not lay down completely flat on the cellulose surface but spread out loops and tails that could interfere with cellulase action.

From the discussion above, it seems that since heteromannans were mixed with cellulose before cellulase, their adsorption on cellulose might have reduced the number of cellulase binding sites and consequently lowered cellulose conversion. However, Figure 8 shows that adding 2.5 g/L GG-GalM to the hydrolysis slurry 4, 24, and 72 h after cellulase addition was still strongly inhibitory, and conversion did not seem to increase much after GG-GalM addition. This data suggest that mere surface adsorption and consequent restriction of cellulose accessibility may not be the only reasons for the drop in conversion. However, research is ongoing in our laboratory to clarify the cause(s) and mechanism(s) for the drop in conversion. Research is also in progress to see the effects of mannan polysaccharides on complexed cellulase system of *Clostridium thermocellum*. On a different note, Han et al. showed that the addition of mannan polysaccharides to cellulose culture repressed cellulase *cbpA* expression for *Clostridium cellulovorans* (Han et al., 2003). Thus, the effects of mannan polysaccharides and their oligomers need to be investigated for other clostridia (such as *C.thermocellum*) and microbial systems as well.

Cellulase inhibition by unsubstituted and less substituted heteromannans was largely overcome by supplementing cellulase with accessory enzymes (Fig. 5a and b). However, this strategy was not very effective for hydrolysis of Avicel mixed with highly substituted heteromannan or its oligomers, that is, GG-GalM (Fig. 5c and Fig. S2). Nonetheless, supplementation of cellulase with purified E-M and Alfa-Gal removed cellulase inhibition by GG-GalM almost completely suggesting that commercial enzyme preparations have insufficient amounts of these enzyme activities to hydrolyze highly substituted mannan.

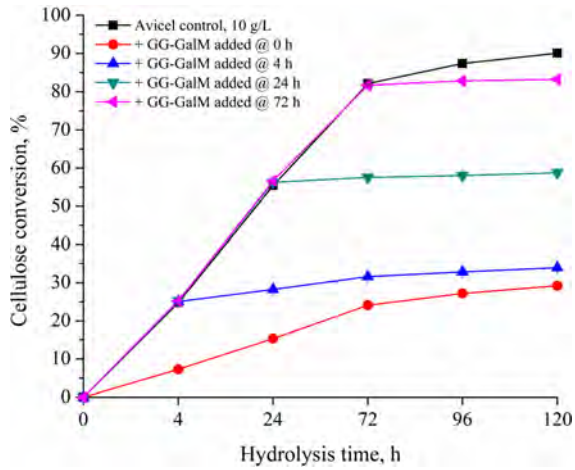

Figure 8. Percentage cellulose conversion versus hydrolysis time (h) for Avicel cellulose (10 g/L) alone and Avicel cellulose mixed with guar gum (GG) galactomannan (GalM; 2.5 g/L) added at various hydrolysis time points of 0, 4, 24, and 72 h. Note: Enzymatic hydrolysis in all cases was performed in a 50 mM sodium citrate buffer at 50°C and 150 rpm at an Accellerase® 1500 cellulase loading of 15 mg protein/g glucan.

Conclusions

Heteromannans were discovered to be strong inhibitors of cellulase, and this inhibition increased with loading and galactose substitution of the mannose backbone. It was also discovered that highly substituted heteromannans significantly dropped cellulose 120 h conversion even at a very low concentration of 0.1 g/L. At the loadings investigated, unhydrolyzed mannan polysaccharides were more inhibitory than their respective enzymatically prepared hydrolyzates (oligomers) except highly substituted mannans. Supplementation of cellulase with commercial accessory enzymes significantly relieved inhibition but did not prove effective for hydrolysis of cellulose mixed with highly substituted mannan such as GG and FGG-GalM. Furthermore, supplementation of cellulase with purified accessory enzymes was shown to completely remove cellulase inhibition by highly substituted mannan. The findings reported herein would greatly help in designing better enzyme cocktails and pretreatments for biomass conversion at high yields.

We gratefully acknowledge support by the Office of Biological and Environmental Research in the DOE Office of Science through the BioEnergy Science Center (BESC). We are thankful to the Center for Environmental Research and Technology (CE-CERT) for providing facilities and equipment used in this research. We would also like to thank the Ford Motor Company for their support of the Chair in Environmental Engineering at the University of California Riverside (UCR).

References

Andrić P, Meyer AS, Jensen PA, Dam-Johansen K. 2010. Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis: I. Significance and mechanism of cellobiose and glucose inhibition on cellulolytic enzymes. Biotechnol Adv 28(3):308–324.

Banerjee G, Car S, Scott-Craig J, Borrusch M, Walton J. 2010a. Rapid optimization of enzyme mixtures for deconstruction of diverse pretreatment/biomass feedstock combinations. Biotechnol Biofuels 3(1):22.

Banerjee G, Car S, Scott-Craig JS, Borrusch MS, Bongers M, Walton JD. 2010b. Synthetic multi-component enzyme mixtures for deconstruction of lignocellulosic biomass. Bioresour Technol 101(23):9097–9105.

Bayer EA, Chanzy H, Lamed R, Shoham Y. 1998. Cellulose, cellulases and cellulosomes. Curr Opin Struct Biol 8(5):548–557.

Berlin A, Maximenko V, Gilkes N, Saddler J. 2007. Optimization of enzyme complexes for lignocellulose hydrolysis. Biotechnol Bioeng 97(2): 287–296.

Beukes N, Chan H, Doi RH, Pletschke BI. 2008. Synergistic associations between Clostridium cellulovorans enzymes XynA, ManA and EngE against sugarcane bagasse. Enzyme Microb Technol 42(6):492–498.

Choi IS, Wi SG, Kim S-B, Bae H-J. 2012. Conversion of coffee residue waste into bioethanol with using popping pretreatment. Bioresour Technol 125(0):132–137.

Clarke JH, Davidson K, Rixon JE, Halstead JR, Fransen MP, Gilbert HJ, Hazlewood GP. 2000. A comparison of enzyme-aided bleaching of softwood paper pulp using combinations of xylanase, mannanase and α-galactosidase. Appl Microbiol Biotechnol 53(6):661–667.

Clayton DW, Phelps GR. 1965. The sorption of glucomannan and xylan on alpha-cellulose wood fibers. J Polym Sci Pol Symp 11(1): 197–220.

Di Risio S, Hu CS, Saville BA, Liao D, Lortie J. 2011. Large-scale, high-solids enzymatic hydrolysis of steam-exploded poplar. Biofuels, Bioprod Bioref 5(6):609–620.

Eronen P, Osterberg M, Heikkinen S, Tenkanen M, Laine J. 2011. Interactions of structurally different hemicelluloses with nanofibrillar cellulose. Carbohydr Polym 86(3):1281–1290.

Gong C-S, Ladisch MR, Tsao GT. 1977. Cellobiase from Trichoderma viride: Purification, properties, kinetics, and mechanism. Biotechnol Bioeng 19(7):959–981.

Gusakov AV, Sinitsyn AP, Gerasimas VB, Savitskene RY, Steponavichus YY. 1985. A product inhibition study of cellulases from Trichoderma longibrachiatum using dyed cellulose. J Biotechnol 3(3):167–174.

Halliwell G, Griffin M. 1973. The nature and mode of action of the cellulolytic component C1 of Trichoderma koningii on native cellulose. Biochem J 135(4):587–594.

Han SO, Yukawa H, Inui M, Doi RH. 2003. Regulation of expression of cellulosomal cellulase and hemicellulase genes in clostridium cellulovorans. J Bacteriol 185(20):6067–6075.

Hannuksela T, Fardim P, Holmbom B. 2003. Sorption of spruce O-acetylated galactoglucomannans onto different pulp fibres. Cellulose 10(4):317–324.

Hannuksela T, Holmbom B. 2003. Sorption of mannans to different fiber surfaces: An evolution of understanding. In: Gatenholm P, Tenkanen M, editors. Hemicelluloses: Science and technology. Washington, DC, USA: American Chemical Society. p 222–235.

Hannuksela T, Tenkanen M, Holmbom B. 2002. Sorption of dissolved galactoglucomannans and galactomannans to bleached kraft pulp. Cellulose 9(3–4):251–261.

Hansson J-Å. 1970. Sorption of hemicelluloses on cellulose fibers. 2. Sorption of glucomannan. Holzforschung 24(3):77.

Henrissat B, Driguez H, Viet C, Schulein M. 1985. Synergism of cellulases from trichoderma reesei in the degradation of cellulose. Nat Biotech 3(8):722–726.

Holtzapple M, Cognata M, Shu Y, Hendrickson C. 1990. Inhibition of Trichoderma reesei cellulase by sugars and solvents. Biotechnol Bioeng 36(3):275–287.

Hong J, Ladisch MR, Gong CS, Wankat PC, Tsao GT. 1981. Combined product and substrate inhibition equation for cellobiase. Biotechnol Bioeng 23(12):2779–2788.

Jørgensen H, Sanadi A, Felby C, Lange N, Fischer M, Ernst S. 2010. Production of ethanol and feed by high dry matter hydrolysis and fermentation of palm Kernel Press Cake. Appl Biochem Biotechnol 161(1–8):318–332.

Kadam KL, Rydholm EC, McMillan JD. 2004. Development and validation of a kinetic model for enzymatic saccharification of lignocellulosic biomass. Biotechnol Progr 20(3):698–705.

Kang L, Lee YY, Yoon SH, Smith AJ, Krishnagopalan GA. 2012. Ethanol production from the mixture of hemicellulose prehydrolysate and paper sludge. BioResources 7(3):3607–3626.

Karimi K, Shafiei M, Kumar R. 2013. Progress in physical and chemical pretreatment of lignocellulosic biomass. In: Gupta VK, Tuohy MG, editors. Biofuel technologies. Berlin, Heidelberg: Springer. p 53–96.

Kim Y, Ximenes E, Mosier NS, Ladisch MR. 2011. Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass. Enzyme Microb Technol 48(4–5):408–415.

Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW. 2012. The challenge of enzyme cost in the production of lignocellulosic biofuels. Biotechnol Bioeng 109(4):1083–1087.

Kont R, Kurasin M, Teugjas H, Valjamae P. 2013. Strong cellulase inhibitors from the hydrothermal pretreatment of wheat straw. Biotechnol Biofuels 6(1):135.

Kristensen J, Felby C, Jorgensen H. 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnol Biofuels 2(1):11.

Kumar L, Chandra R, Chung PA, Saddler J. 2010. Can the same steam pretreatment conditions be used for most softwoods to achieve good, enzymatic hydrolysis and sugar yields? Bioresour Technol 101(20):7827–7833.

Kumar R, Hu F, Sannigrahi P, Jung S, Ragauskas AJ, Wyman CE. 2013. Carbohydrate derived-pseudo-lignin can retard cellulose biological conversion. Biotechnol Bioeng 110(3):737–753.

Kumar R, Mago G, Balan V, Wyman CE. 2009. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. Bioresour Technol 100(17):3948–3962.
Kumar R, Wyman CE. 2008. An improved method to directly estimate cellulase adsorption on biomass solids. Enzyme Microb Technol 42(5): 426–433.
Kumar R, Wyman CE. 2009a. Effect of additives on the digestibility of corn stover solids following pretreatment by leading technologies. Biotechnol Bioeng 102(6):1544–1557.
Kumar R, Wyman CE. 2009b. Effect of enzyme supplementation at moderate cellulase loadings on initial glucose and xylose release from corn stover solids pretreated by leading technologies. Biotechnol Bioeng 102(2): 457–467.
Kumar R, Wyman CE. 2009c. Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies. Bioresour Technol 100(18):4203–4213.
Kumar R, Wyman CE. 2009d. Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies. Biotechnol Progr 25(2):302–314.
Kumar R, Wyman CE. 2010. Key features of pretreated lignocelluloses biomass solids and their impact on hydrolysis. In: Waldon K, editor. Bioalcohol production: Biochemical conversion of lignocellulosic biomass. Oxford: Woodhead publishing limited. p 73–121, invited.
Kumar R, Wyman CE. 2013. Physical and chemical features of pretreated biomass that influence macro-/micro-accessibility and biological processing. In: Wyman CE, editor. Aqueous pretreatment of plant biomass for biological and chemical conversion to fuels and chemicals. John Wiley & Sons, Ltd. p 281–310.
Li H, Pu Y, Kumar R, Ragauskas AJ, Wyman CE. 2014. Investigation of lignin deposition on cellulose during hydrothermal pretreatment, its effect on cellulose hydrolysis, and underlying mechanisms. Biotechnol Bioeng 111(3):485–492.
Lloyd T, Wyman CE. 2003. Application of a depolymerization model for predicting thermochemical hydrolysis of hemicellulose. Appl Biochem Biotechnol 105–108:53–67.
Lynd LR, Cushman JH, Nichols RJ, Wyman CE. 1991. Fuel ethanol from cellulosic biomass. Science (Washington, DC) 251(4999):1318–1323.
Lynd LR, Laser MS, Bransby D, Dale BE, Davison B, Hamilton R, Himmel M, Keller M, McMillan JD, Sheehan J, Wyman CE. 2008. How biotech can transform biofuels. Nat Biotechnol 26(2):169–172.
Lynd LR, Wyman CE, Gerngross TU. 1999. Biocommodity engineering. Biotechnol Progr 15(5):777–793.
Mandels M, Reese ET. 1957. Induction of cellulases in trichoderma viride as influenced by carbon sources and metals. J Bacteriol 73(2):269–278.
Mishima T, Hisamatsu M, York WS, Teranishi K, Yamada T. 1998. Adhesion of β-d-glucans to cellulose. Carbohydr Res 308(3–4):389–395.
Newman RH, Hemmingson JA. 1998. Interactions between locust bean gum and cellulose characterized by13C n.m.r. spectroscopy. Carbohydr Polym 36(2/3):167–172.
Parikka K, Leppänen A-S, Xu C, Pitkänen L, Eronen P, Österberg M, Brumer H, Willför S, Tenkanen M. 2012. Functional and anionic cellulose-interacting polymers by selective chemo-enzymatic carboxylation of galactose-containing polysaccharides. Biomacromolecules 13(8):2418–2428.
Penttilä PA, Várnai A, Pere J, Tammelin T, Salmén L, Siika-aho M, Viikari L, Serimaa R. 2013. Xylan as limiting factor in enzymatic hydrolysis of nanocellulose. Bioresour Technol 129(0):135–141.
Qing Q, Yang B, Wyman CE. 2010. Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. Bioresour Technol 101(24):9624–9630.
Reese ET. 1957. Biological degradation of cellulose derivatives. Ind Eng Chem 49(1):89–93.
Selig M, Weiss N, Ji Y. 2008. Enzymatic saccharification of lignocellulosic biomass. Golden, CO, USA: National Renewable Energy Laboratory.
Shallom D, Shoham Y. 2003. Microbial hemicellulases. Curr Opin Microbiol 6(3):219–228.
Sinner M, Parameswaran N, Dietrichs HH. 1979. Degradation of delignified sprucewood by purified mannanase, xylanase, and cellulases. In: Brown RD Jr., Jurasek L, editors. Hydrolysis of cellulose: Mechanisms of enzymatic and acid catalysis. Washington, DC, USA: American Chemical Society. p 303–329.
Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D. 2008. Determination of structural carbohydrates and lignin in biomass. Laboratory Analytical Procedures (LAPs). Golden, CO: National Renewable Energy Laboratory.
Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. Anal Biochem 150(1):76–85.
Stahlberg J, Johansson G, Pettersson G. 1991. A new model for enzymatic hydrolysis of cellulose based on the two-domain structure of cellobiohydrolase I. Nat Biotech 9(3):286–290.
Stahlberg J, Johansson G, Pettersson G. 1993. Trichoderma reesei has no true exo-cellulase: All intact and truncated cellulases produce new reducing end groups on cellulose. Biochim Biophys Acta 1157(1):107–113.
Tenkanen M, Buchert J, Viikari L. 1995. Binding of hemicellulases on isolated polysaccharide substrates. Enzyme Microb Technol 17(6):499–505.
Teugjas H, Valjamae P. 2013. Product inhibition of cellulases studied with 14C-labeled cellulose substrates. Biotechnol Biofuels 6(1):104.
Tucker M, Nagle N, Jennings E, Ibsen K, Aden A, Nguyen Q, Kim K, Noll S. 2004. Conversion of distiller's grain into fuel alcohol and a higher-value animal feed by dilute-acid pretreatment. Appl Biochem Biotechnol 115(1–3):1139–1159.
Varnai A, Huikko L, Pere J, Siika-aho M, Viikari L. 2011. Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood. Bioresour Technol 102(19):9096–9104.
Whitney SEC, Brigham JE, Darke AH, Reid JSG, Gidley MJ. 1998. Structural aspects of the interaction of mannan-based polysaccharides with bacterial cellulose. Carbohydr Res 307(3–4):299–309.
Wyman CE. 1994. Ethanol from lignocellulosic biomass—Technology, economics, and opportunities. Bioresour Technol 50(1):3–16.
Wyman CE, Decker SR, Himmel ME, Brady JW, Skopec CE, Viikari L. 2005. Hydrolysis of cellulose and hemicellulose. In: Dumitriu S, editor. Polysaccharides: Structural diversity and functional versatility. NY: Marcel Dekker, Inc. p 995–1033.
Ximenes E, Kim Y, Mosier N, Dien B, Ladisch M. 2011. Deactivation of cellulases by phenols. Enzyme Microb Technol 48(1):54–60.
Yang B, Wyman CE. 2006. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. Biotechnol Bioeng 94(4): 611–617.
Yang B, Wyman CE. 2008. Pretreatment: The key to unlocking low-cost cellulosic ethanol. Biofuels, Bioprod Bioref 2(1):26–40.
Zhang J, Siika-aho M, Tenkanen M, Viikari L. 2011. The role of acetyl xylan esterase in the solubilization of xylan and enzymatic hydrolysis of wheat straw and giant reed. Biotechnol Biofuels 4(1):60.
Zhang YHP, Himmel ME, Mielenz JR. 2006. Outlook for cellulase improvement: Screening and selection strategies. Biotechnol Adv 24(5):452–481.
Zhang Z, Donaldson AA, Ma X. 2012. Advancements and future directions in enzyme technology for biomass conversion. Biotechnol Adv 30(4): 913–919.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.