Effects of Cellulase and Xylanase Enzymes on the Deconstruction of Solids from Pretreatment of Poplar by Leading Technologies

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Comparative data is presented on glucose and xylose release for enzymatic hydrolysis of solids produced by pretreatment of poplar wood by ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), controlled pH, dilute acid, flowthrough (FT), lime, and sulfur dioxide (SO_2) technologies. Sugar solubilization was measured for times of up to 72 h using cellulase supplemented with β-glucosidase at an activity ratio of 1:2, respectively, at combined protein mass loadings of 5.8–116 mg/g of glucan in poplar wood prior to pretreatment. In addition, the enzyme cocktail was augmented with up to 11.0 g of xylanase protein per gram of cellulase protein at combined cellulase and β-glucosidase mass loadings of 14.5 and 29.0 mg protein (about 7.5 and 15 FPU, respectively)/g of original potential glucose to evaluate cellulase–xylanase interactions. All pretreated poplar solids required high protein loadings to realize good sugar yields via enzymatic hydrolysis, and performance tended to be better for low pH pretreatments by dilute sulfuric acid and sulfur dioxide, possibly due to higher xylose removal. Glucose release increased nearly linearly with residual xylose removal by enzymes for all pretreatments, xylanase leverage on glucan removal decreased at high cellulase loadings. Washing the solids improved digestion for all pretreatments and was particularly beneficial for controlled pH pretreatment. Furthermore, incubation of pretreated solids with BSA, Tween 20, or PEG6000 prior to adding enzymes enhanced yields, but the effectiveness of these additives varied with the type of pretreatment. © 2009 American Institute of Chemical Engineers *Biotechnol. Prog.*, 25: 302–314, 2009
Keywords: pretreatment, enzymatic hydrolysis, cellulase, xylanase, xylanase leverage, additives, poplar wood

Introduction

Ethanol production from corn and other starch crops is growing very rapidly in the United States of late, reaching over 7.6 billion gallons of ethanol capacity as of January 2008, mostly for use as a gasoline additive (http://www1.eere.energy.gov/biomass). Currently, starch ethanol substitutes for over 5.0% of gasoline by volume, and ethanol production from starch is expected to grow further.[1–3] However, to make a significant impact on our dependence on petroleum imports from unstable regions of the world and reduce emissions of greenhouse gases from the transportation sector, it is vital to introduce production ethanol and other liquid fuels from such cellulosic biomass sources as (1) paper and other municipal solid wastes; (2) wheat straw, rice straw, corn stover, rice hulls, soybean hulls, and other agriculture residues; (3) sawdust, tree thinnings, and other forestry wastes; (4) industrial wastes such as paper sludge; (5) herbaceous energy crops including miscanthus, alfalfa, switchgrass, and red canary grass; and (6) woody energy crops such as willow (Salix spp.), eucalyptus (Eucalyptus spp.), aspen, and hybrid poplar (Populus spp.).[4–7] These materials are more difficult to convert to fermentation sugars than starch crops and must be pretreated to realize the high ethanol yields vital to low costs.[8]

After trying numerous approaches, a few promising pretreatment technologies have emerged over the years,[9,10] but comparative data is essential to determine how these pretreatments perform with different feedstocks. Therefore, a Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) was formed among pretreatment experts to provide comparative data on leading options for the first time. In the first CAFI study supported by the US Department of Agriculture, ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), controlled pH, dilute sulfuric acid (DA), lime, and liquid flowthrough pretreatments all proved very effective in achieving high sugar yields from corn stover, with AFEX giving slightly higher total sugar yields. This article reports on the rate of enzyme catalyzed release of glucose and xylose from solids produced by application of the same set of pretreatment

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Table 1. Pretreatment Conditions, Compositions, and Yields of glucan and xylan from Solids Prepared by Leading Technologies

Pretreatment	Pretreatment Conditions	Pretreatment Severity*, log R_0	Yield of Component Left in Pretreated Solids (%)		Composition of Pretreated Solids (%)		
			Glucan†	Xylan‡	Glucan	Xylan	Lignin
Untreated	NA	NA	NA		43.8	14.8	29.1
AFEX	180°C,700 psi, 2:1 NH_3 to Biomass, 30 min, and 233% moisture content (dwb)-NW	3.83	100	100	46.6	15	nd
ARP	185°C,400 psi, 3.66:1 NH_3 to Biomass (dry wt), 27.5 min and 23.49% solid-W	3.94	93.2	68.2	57.5	13.5	24.8
Controlled pH	200°C, 10 min (+5 min heat up)-NW	3.94	98.0	42.1	58.8	7.0	32.2
Dilute acid	POP-1 190°C, 0.02 H_2SO_4:Dry wt, 70 s, 30% solids-NW	2.72	87.9	8.3	57.3	2.1	46.1
	POP-2 190°C, 0.02 H_2SO_4:Dry wt, 120 s, 30% solids-NW	2.95	88.0	7.9	60.2	1.8	42.5
	POP-3 200°C, 0.02 H_2SO_4:Dry wt, 70 s, 30% solids-NW	3.01	75.7	5.4	54.6	1.5	49.1
	POP-4 200°C, 0.02 H_2SO_4:Dry wt, 120 s, 30% solids-NW	3.25	67.0	2.9	44.3	0.7	59.3
Flowthrough	190°C, 0.05% H_2SO_4, 20 mL/min, 15 min	3.83	80.2	5.7	65.6	1.6	33.7
Lime	65°C, 0.5:1 $Ca(OH)_2$ to Biomass (dry wt)-W1	3.57	98.1	96.2	53.1	16.8	18.0
SO_2	200°C, 5 min, 3% SO_2-steam explosion-NW	3.64	96.9	9.3	55.1	2.5	nd

* Severity parameters includes only time and temperature; log R_0 = log [time exp($(H - R)$/14.75)], where time is in minutes, H is the pretreatment temperature in °C, R is a reference temperature, 100°C. † Glucan defined in terms of $C_6H_{10}O_5$. ‡ Xylan defined in terms of $C_5H_8O_4$.

technologies plus sulfur dioxide to a woody biomass, poplar, through the support of the Office of the Biomass Program of the US Department of Energy. Cellulase enzyme supplemented with a fixed ratio of β-glucosidase was applied over a range of total protein loadings, and the impact of adding different amounts of xylanase to enhance removal of residual xylose was measured at two of the resulting combined cellulase/β-glucosidase mass loadings. In addition, the effects of washing the solids prior to enzymatic hydrolysis and treatment with BSA, Tween 20, or PEG 6000 were evaluated.

Materials and Methods

Substrates

Pure cellulose, Avicel PH-101, was purchased from FMC Corporation, Philadelphia, PA (Cat 11365, Lot 1094627); bovine serum albumin (BSA, Cat A9056) from Sigma Chemicals, St. Louis, MO; and Tween 20 (Cat AC23336-2500, Lot # A0226412) and PEG6000 (Cat NC9166418, Lot # 1370757) from Fisher Scientific, Pittsburgh, PA. Birchwood and beechwood xylans were purchased from Sigma Chemicals, St. Louis, MO. Unpretreated poplar was generously provided by the National Renewable Energy Laboratory (NREL) in Golden, CO. Solids prepared by pretreatment of poplar by ARP, AFEX, controlled pH, lime, and SO_2 were generously provided by our CAFI partners from Auburn University, Michigan State University, Purdue University, Texas A&M University, and the University of British Columbia, respectively. The National Renewable Energy Laboratory (NREL) provided solids prepared at four different conditions via dilute acid technology using their steam explosion equipment. The pretreatment conditions and solids compositions as determined by NREL Laboratory Analytical Procedure 002[11] are reported in Table 1. It is important to recognize that continued development of these pretreatments can result in further improvements in performance beyond that reported here.

Enzymes

Spezyme® CP cellulase (Lot 301-04075-034; 59 ± 5 FPU/mL, 123 ± 10 mg protein/mL), Multifect® Xylanase (Lot 301-04021-015; 42 ± 5 mg protein/mL), and β-glucosidase (31 ± 5 mg protein/mL) and measurements of their protein content/activities were generously provided by Genencor Division of Danisco US, (Rochester, NY, USA). The enzyme protein contents were determined by the standard BCA method.[12]

Enzymatic hydrolysis

Consistent with NREL Laboratory Analytical Procedure LAP009,[13] hydrolysis experiments were performed in at least duplicates at 1% (w/v) glucan equivalent concentrations in 0.05 M citrate buffer containing antibiotics (400 μL per 100 mL of 10 mg/mL tetracycline in 70% ethanol, and 300 μL per 100 mL of 10 mg/mL cyclohexamide in DI water) in 125-mL Erlenmeyer flasks at 48°C ± 3°C as controlled by a thermostated water shaker unit operated at ~200 rpm. Similarly, substrate blanks without enzyme and enzyme blanks without substrate were run in parallel. Digestibility was determined at cellulase (Spezyme CP) mass loadings of 5.8, 14.5, 29.0, and 116.1 mg of protein per g glucan equivalent in the raw biomass (corresponding to about 3.0, 7.5, 15, and 60 FPU/g original* glucan) supplemented with β-glucosidase at a CBU to FPU ratio of ~2 or a protein mass ratio of 0.034. However, solids pretreated with dilute acid at four different conditions were screened at a fixed enzyme loading of ~97 mg protein/g original glucan (about 50 FPU) with a CBU:FPU activity ratio of ~2, and further digestibility experiments were run with only the solids giving the highest total sugar yields via this screening approach. The digestibility of Avicel and selected solids were also determined with GC 220 cellulase using the same protein loadings as for Spezyme CP.

For xylanase supplementation, the loadings were based on xylanase-to-cellulase protein mass ratios of 0.2, 0.5, 1, 2, and 5 with a combined cellulase/β-glucosidase (CAB†) loading of 29.0 mg protein/g original glucan (about 15 FPU/g original glucan). To see whether modifying the enzyme cocktail would reduce the enzyme loadings needed to achieve similar yields, the impact of xylanase supplementation was further studied at a lower CAB mass loading of 14.5 mg protein/g original glucan (about 7.5 FPU/g original glucan). The same xylanase to cellulase protein ratios, plus

*g of glucan in poplar wood prior to pretreatment.
†Cellulase added with beta-glucosidase (CAB).

Table 2. Total Combined Amounts of Cellulase, β-Glucosidase, and Xylanase Protein Per Gram Glucan in Unpretreated Poplar for Various Xylanase to Cellulase Protein Ratios

	Total Amount of Protein (mg/g Original Glucan)	
Xylanase to Cellulase Protein Mass Ratio	14.5 mg Cellulase (~7.5 FPU)	29.0 mg Cellulase (~15 FPU)
0.0:1	14.5	29.0
0.2:1	16.8	33.7
0.5:1	21.1	42.1
1:1	28.1	56.1
2:1	42.1	84.2
5:1	84.2	168.4
11:1	168.4	NA

an additional ratio of 11, were used as for the higher cellulase loading. The xylanase loadings were designated as $MX_{0.2}$, $MX_{0.5}$, MX_1, MX_2, MX_5, and MX_{11} for xylanase to cellulase protein mass ratios of 0.2, 0.5, 1, 2, 5, and 11, respectively, unless otherwise stated. The total amounts of protein for each cellulase and xylanase loading are summarized in Table 2.

To determine the rate of sugar generation by enzymatic hydrolysis, liquid samples of about 700 μL were withdrawn at 24, 48, and 72 h, then immediately filtered through 0.2-μm nylon filter vials (Alltech Associates, Deerfield, IL), pipetted into 500 μL polyethylene HPLC vials (Alltech Associates, Deerfield, IL), and kept refrigerated at 4°C (frozen at −20°C for longer storage) until analyzed. The hydrolysis samples along with calibration standards were run on a Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA) employing Aminex HPX-87H and HPX-87P columns (Bio-Rad Laboratories, Life Science Research, Hercules, CA). Hydrolysis for longer times could improve yields beyond those reported here.

Xylanase addition prior to cellulase

To determine the impact of adding xylanase prior to cellulase on cellulase efficacy, pretreated solids for all pretreatment were incubated at 50°C with just xylanase at a loading of 28 mg/g original glucan (MX_2) for 24 and 72 h. After 24 or 72 h of hydrolysis with just xylanase, cellulase was added at a loading of 14.5 mg/g original glucan (about 7.5 FPU/g glucan) with β-glucosidase supplementation at a CBU: FPU ratio of ~2. The samples taken before cellulase addition were designated to be at 0 h.

Impact of additives

For solids showing relatively high rates of sugar release, the impact on solids digestibility of adding the noncatalytic protein BSA, the surfactant Tween20, or the polymer PEG6000 at a loading of 300 mg/g glucan (3.0 mg/mL) was determined. Pretreated poplar solids were incubated with one of these additives at hydrolysis temperature for at least 4 h prior to enzyme addition. Samples were drawn at 24-h intervals, and a small amount (~30 mg) of the resin AG50W-X8 (Bio-Rad Laboratory, Hercules, CA, Cat 143-5441) was added to the samples having surfactant and polymer to minimize damage to the HPLC and guard columns and especially the Aminex HPX-87P column.

Yield calculations

The yields of xylose and glucose reported below were calculated in two ways. For yields from just enzymatic hydrolysis, the amount of glucose or xylose in solution following just enzymatic hydrolysis was compared with the glucan and xylan left in the solids after pretreatment. Thus, for glucose from just enzymatic hydrolysis, the appropriate formula is:

$$\text{glucose yield}^{EH} = [\text{glucose in EH liquid sample (g)} + 1.053 \times \text{cellobiose in EH liquid sample (g)}]/1.111 \times \text{initial amount of glucan to EH (g)}$$

For xylose from just enzymatic hydrolysis, the calculation is:

$$\text{xylose yield}^{EH} = \text{xylose in EH liquid sample (g)}]/1.136 \times \text{initial amount of xylan to EH (g)}$$

Total yields were also calculated from the amount of glucose or xylose in solution from both pretreatment (PR) and enzymatic hydrolysis (EH) compared with the amount of glucan and xylan available in the unpretreated biomass. Thus, for glucose, the total yield was determined as:

$$\text{total glucose yield (TGY)} = \text{glucose yield}^{PR} + \text{glucose yield}^{EH} \times \text{pretreatment yield of glucan (amount of glucan left in solids)}$$

For xylose, the total yields according to this approach are:

$$\text{total xylose yield (TXY)} = \text{xylose yield}^{PR} + \text{xylose yield}^{EH} \times \text{pretreatment yield of xylan (amount of xylan left in solids)}$$

Finally, the overall sugar yields for both xylose and glucose were determined for poplar as:

$$\text{total sugar yield (TSY)} = (\text{maximum potential glucose [48.66]} \times \text{TGY} + \text{maximum potential xylose [16.93]} \times \text{TXY})/\text{maximum potential glucose plus xylose [65.58]}$$

Results

Comparison of total yields for dilute acid pretreated solids

As shown in Figure 1, glucose yield data for duplicate runs after 72 h of hydrolysis with 97 mg protein/g original glucan (equivalent to about 50 FPU/g glucan) revealed that solids pretreated with dilute sulfuric acid at all four conditions in the NREL steam gun were reasonably digestible. However, the digestibilities of POP-1[‡] and POP-3 solids were higher after 72 h than for POP-2 and POP-4, and POP-1 gave slightly higher total 72 h sugar yields from the combined operations of pretreatment and enzymatic hydrolysis. Therefore, further experiments for dilute acid pretreatment focused on just the POP-1 solids.

Yields vs. protein loadings

To understand the impact of cellulase loadings on glucose and xylose release, xylose and glucose yield data were determined from averages of duplicates after 72 h of hydrolysis vs. the total protein added. Because enzymes are very expensive, ethanol production costs are most sensitive to the total amount of protein used, and the amount of sugars produced per mg of protein indirectly represents the effectiveness of pretreatment. Thus, enzyme use should be minimized to make cellulosic ethanol cost competitive with conventional fuels.[14,15] Accordingly, Figures 2 and 3 show the effects of protein loadings on glucose and xylose yield, respectively. At the lower protein loading of 5.8 mg, glucose yields for

[‡]POP-1, 2, 3, and 4 are dilute acid pretreated poplar solids prepared at different conditions, as described in Table 1.

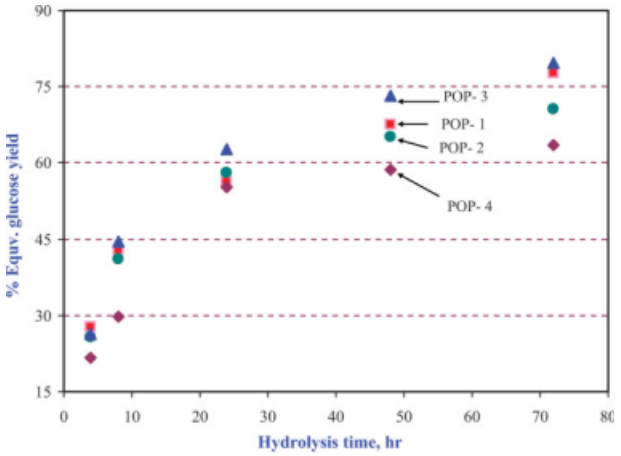

Figure 1. Equivalent glucose yields vs. time at 97 mg/g original glucan (about 50 FPU/g original glucan) for poplar solids pretreated with dilute acid at the four conditions described in the materials and methods section.

POP-1, 2, 3, and 4 are dilute acid pretreated poplar solids prepared at different conditions, as described in Table 1.

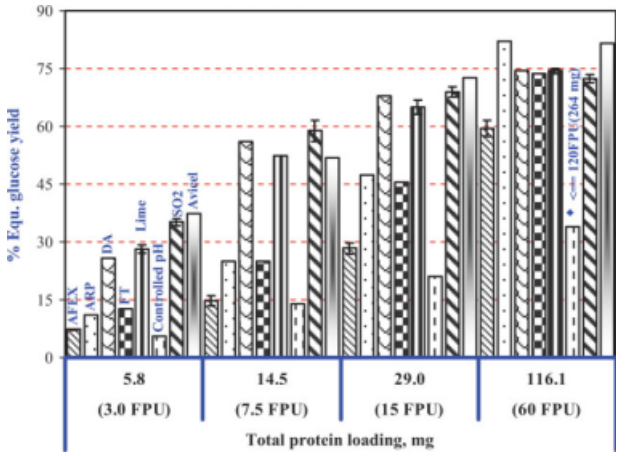

Figure 2. Equivalent glucose yields vs. mass protein loadings after 72 h of digestion for pure Avicel glucan and poplar solids prepared by leading pretreatment technologies. Equivalent glucose = (glucose+1.053 × cellobiose).

◆ Equivalent glucose yield for controlled pH at a cellulase added with β-glucosidase mass loading of 264 mg (120 FPU).

all pretreated solids of poplar were a little lower than for Avicel pure cellulose, as showed in Figure 2. SO_2 solids showed the fastest digestion at lower protein loadings (>30%). However, at the highest protein loadings, corresponding to 60 FPU, glucose yields for all solids were nearly identical at more than 74% and comparable to yields from pure cellulose except for controlled pH (~32%) and AFEX solids (~60%). Furthermore, controlled pH pretreatment showed much slower rates of digestion even at a high protein loading of ~264 mg.

Because dilute acid, flowthrough, and SO_2 pretreated poplar left very little xylose in the solids, the release of xylose from the solids resulting from these three pretreatments was almost 100% at a very low combined cellulase/β-glucosidase protein loading of 14.5 mg protein/g original glucan. Xylose release was also very high for lime poplar solids, especially at low protein loadings, and comparable to that for pure xylans derived from beechwood and birchwood. The enhanced removal of xylose from lime pretreated solids may result from greater deacetylation of biomass by this approach.[16,17] Furthermore, the binding of xylan with fiber and its degree of substitution may also limit its accessibility.[18–20] At high protein loadings, xylose yields for all pretreated solids except controlled pH were nearly equal.

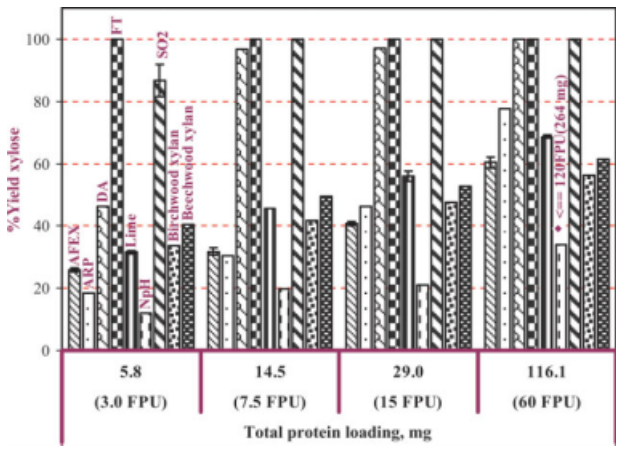

Figure 3. Xylose yields vs. total protein loadings after 72 h of digestion for pure xylan and poplar solids prepared by leading pretreatment technologies.

Effect of washing on digestibility

In general, thermochemical pretreatments tend to generate soluble sugars, degradation products of sugars and lignin, various other soluble components, and some acids.[21–23] These compounds may be inhibitory to cellulase and other enzymes' catalytic action[24–29] as well as binding.[30,31] For dilute acid and AFEX pretreated solids, hot and cold water washing after pretreatment has been reported to significantly increase digestion by enzymes.[26,32,33] Therefore, the effect of washing on enzymatic hydrolysis was evaluated for AFEX, dilute acid, controlled pH, and SO_2 solids, whereas ARP, lime, and flowthrough solids were received already washed. AFEX and dilute acid solids were washed with DI water (WW) and hot DI water (HWW) in two steps with the total water volume equal to 30 times the wet weight of the biomass. As per a protocol employed by the University of British Columbia, SO_2 pretreated solids were washed with DI water in three steps with the total volume of water equal to 30 times of the total wet weight of biomass. Controlled pH pretreated poplar solids washed with hot water right after the pretreatment were generously provided by Purdue University.

As shown in Figure 4, washing the solids significantly enhanced glucose and xylose release from pretreated solids. Washing with room temperature (RT) or hot water (HW) increased glucose release by 19.7% (RT) and 29.1% (HW) for AFEX, 18.8% (RT) and 23.2% (HW) for dilute acid, 75.4% (HW) for controlled pH, and 28.2% (RT) for SO_2 poplar solids. Enhancement of xylose release after washing with room temperature water or hot water was of 0% (RT) and 10.0% (HW) for AFEX, 5.3% (RT) and 5.3% (HW) for dilute acid, 139.4% (HW) for controlled pH, and 0.0% (RT) for SO_2 pretreated poplar solids.

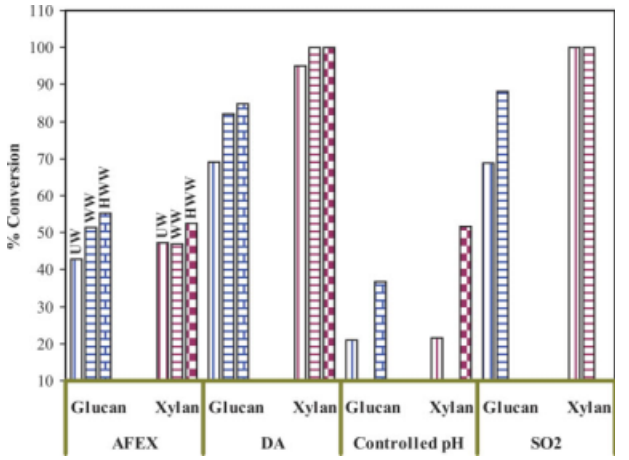

Figure 4. Effect of water washing on glucose and xylose release after 72 h of enzymatic hydrolysis at a CAB mass loading of 29.5 mg/g original glucan for AFEX, dilute acid, controlled pH, and SO_2 pretreated poplar solids.

UW, unwashed; WW, deonized water washed; HWW, hot deionized water washed.

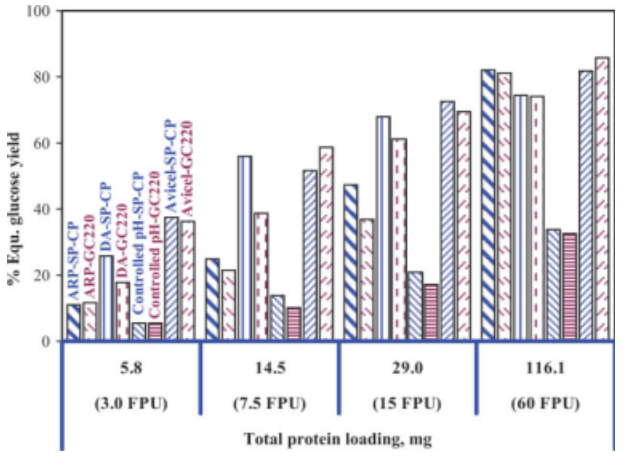

Figure 5. Comparison of glucose release following 72 h of enzymatic hydrolysis of pretreated poplar solids and Avicel glucan with Spezyme and GC220 cellulase.

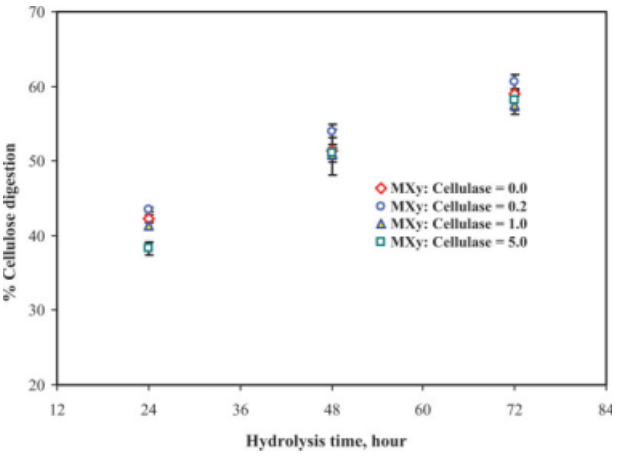

Figure 6. Glucose release vs. time for 72 h hydrolysis of Avicel cellulose at a cellulase loading of 14.5 mg/g glucan with different levels of xylanase supplementation.

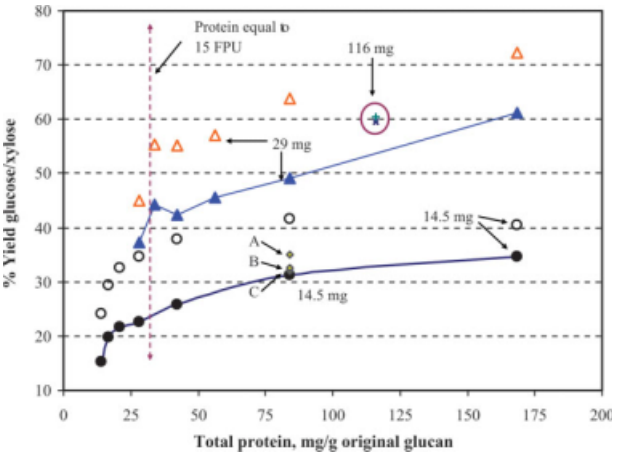

Figure 7. Glucose and xylose yields after 72 h of hydrolysis vs. total protein loadings for xylanase supplementation of constant CAB mass loadings of 29.0 and 14.5 mg/g original glucan for AFEX pretreated poplar solids.

Closed symbols and × symbols show glucose yields and open symbols and + symbols designate xylose yields. A, B, and C represent 14.5 mg + MX_5 + Tween 20, BSA, and PEG 6000, respectively, for a loading of 300 mg/g glucan (3.0 mg/mL).

Spezyme cellulase vs. GC 220

Poplar solids digestibilities were evaluated with another cellulase, GC 220, using the same protein loadings as for Spezyme CP. As shown in Figure 5, there was little difference between the performance of Spezyme CP and GC 220 at high protein loadings for poplar solids and Avicel cellulose. However, Spezyme CP seemed to perform better than GC 220 at lower protein loadings even though GC 220 had higher cellulase, β-glucosidase, and xylanase activity per mL,[34,35] possibly due to a higher protein to FPU ratio (2.1) for Spezyme compared with GC220 (2.0).

Impact of xylanase supplementation

Supplementation of cellulase with xylanase has been reported to enhance glucose release from pretreated biomass solids.[36–38] Studies reported in the literature showed that glucose release is enhanced by xylose removal,[39–46] and it has been argued that removing the hemicellulose coating on cellulose and/or its linkages with cellulose increases cellulose accessibility. However, Weimer et al.[47] suggested that intimate association of hemicellulose and cellulose does not inhibit cellulose digestion. In this study, the effect of xylanase supplementation on glucose and xylose release was studied for a CAB loading of 29.0 mg/g original glucan. As in Figure 6, an insignificant increase in glucose release from Avicel cellulose with xylanase supplementation indicated little cellulase activity for the Multifect xylanase enzyme used in this study, and cellulose digestibility was less than 1 and 2% using xylanase alone at protein loadings corresponding to MX_2 and MX_{11}, respectively (data not shown). To evaluate whether cellulase loadings could be reduced further while achieving high yields, the impact of adding xylanase for a

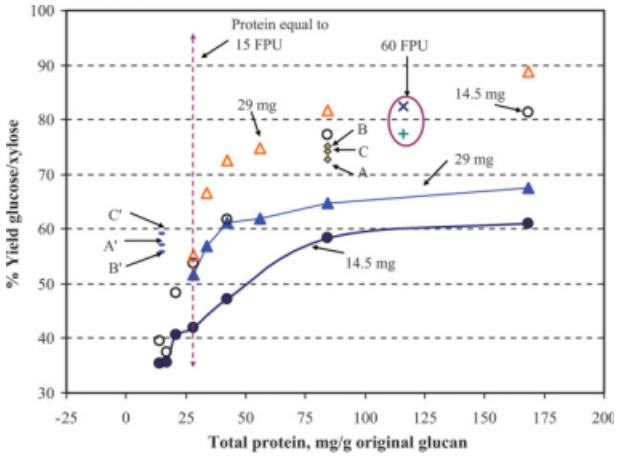

Figure 8. Glucose and xylose yields after 72 h of hydrolysis vs. total protein loadings for xylanase supplementation of constant CAB mass loadings of 29.0 and 14.5 mg/g original glucan for ARP pretreated poplar solids.

Closed symbols and × show avg. glucose yields and open symbol and "+" avg. xylose yields. A′ (A), B′ (B), and C′ (C) represent 14.5 mg (+MX_5) + Tween 20, BSA, and PEG 6000, respectively, at a loading of 300 mg/g glucan (3.0 mg/mL).

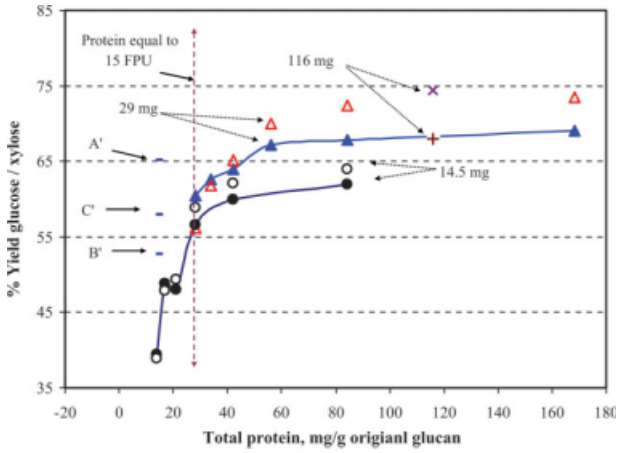

Figure 9. Glucose and xylose yields after 72 h of hydrolysis vs. total protein loadings for xylanase supplementation of constant CAB mass loadings of 29.0 and 14.5 mg/g original glucan for lime pretreated poplar solids.

Closed symbols and × show avg. glucose yields and open symbol and "+" avg. xylose yields. A′, B′, and C′ represent 14.5 mg + Tween 20, BSA, and PEG 6000, respectively, at a loading of 300 mg/g glucan (3.0 mg/mL).

lower CAB loading of 14.5 mg was studied for solids having higher amounts of residual xylose.

Higher Residual Xylan Substrates

AFEX pretreated poplar solids. The effect of xylanase supplementation on glucose and xylose release after 72 h of enzymatic hydrolysis is shown in Figure 7 for poplar solids resulting from AFEX pretreatment. A very high leverage of xylanase on cellulase effectiveness was observed, and short-term glucose and xylose yields increased with increasing xylanase supplementation. Glucan conversion was almost doubled (from 15 to 34% and 37 to 61% at 14.5 and 29.0 mg/g original glucan, respectively) at a xylanase to cellulase mass ratio of 5 (MX_5). Yields of glucose and xylose were higher at a CAB loading of 29.0 mg/g original glucan with increasing xylanase than for 14.5 mg with xylanase supplementation. The glucose and xylose yields shown in Figure 7 for a high CAB loading of 116.1 mg/g original glucan (∼60 FPU) were only a little higher than for 29.0 mg which were in turn much higher than for 14.5 mg. Xylose yields at moderate loadings of 29.0 mg/g original glucan were almost as high as for much greater loadings of 116.1 mg/g original glucan but much higher than for the lower loading of 14.5 mg. Furthermore, Tween20, PEG6000, and BSA increased 72 h glucose/xylose yields by 12.2%/7.7%, 4.2%/6.6%, and 2.6%/negligible, respectively, for a CAB loading at 14.5 mg and a xylanase supplementation mass ratio of 5 (MX_5). However, much greater enhancements of about 65 to 70% and 25 to 30% in 72 h glucose and xylose yields, respectively, were observed with all the additives when used with cellulase alone (data not shown).

ARP pretreated poplar solids. For ARP pretreated solids, the increase in glucose/xylose release with xylanase supplementation was about 25%/40% and 16%/38% for CAB protein loadings of 14.5 and 29.0 mg/g original glucan, respectively. The optimum loading of xylanase seemed to be at a mass ratio of 5 to 1 (MX_5) for both CAB mass loadings. Additives greatly improved 72 h glucose release from ARP poplar solids, as shown in Figure 8. For a CAB mass loading of 14.5 mg, the total enhancement by xylanase supplementation of glucose and xylose release was about 65 and 90%, respectively, and by xylanase and additives together was >105% and >110%, respectively, (xylan data not shown). However, the enhancement in glucose release by additives was greater, >55%, with cellulase alone. Glucose and xylose yields for a CAB loading of 14.5 mg with a xylanase mass supplementation ratio of 5 (MX_5) and use of additives were similar to those obtained for a much higher CAB mass loading of 116.1 mg. All of the additives used with ARP poplar solids gave similar results, and the release of sugars did not seem to increase for loadings greater than 300 mg/glucan (data not shown).

Lime pretreated poplar solids. For lime pretreated poplar solids, the enhancement in glucose release was ∼57% greater at a CAB mass loading of 14.5 mg with xylanase supplementation MX_5 than the 12% increase for a CAB mass loading of 29.0 mg/g original glucan with MX_5, as shown in Figure 9. Furthermore, the ∼29% of xylose released for a CAB mass loading of 29.0 mg with xylanase supplementation MX_5 was lower than the ∼59.7% result with a CAB loading of 14.5 mg with xylanase supplementation MX_5. Thus it appears that the mass loading of CAB affects glucose and xylose release. Tween 20, PEG6000, and BSA additives increased glucose/xylose release by 55%/33.5%, 38%/18.3%, and 25%/17.3%, respectively, at a CAB mass loading at 14.5 mg.

Controlled pH pretreated poplar solids. Hot DI water washed solids produced by controlled pH pretreatment of poplar showed greater than a 70% increase in xylose release and more than a 75% increase in glucose release at a CAB loading of 29.0 mg/g original glucan with xylanase supplementation, as shown in Figure 10. Furthermore, glucose release was almost as high for 29.0 mg of CAB as for the much higher loading of 116.1 mg with just CAB. Yet xylose release was significantly higher for the highest CAB loading. Sugar release at lower CAB mass loading was not measured,

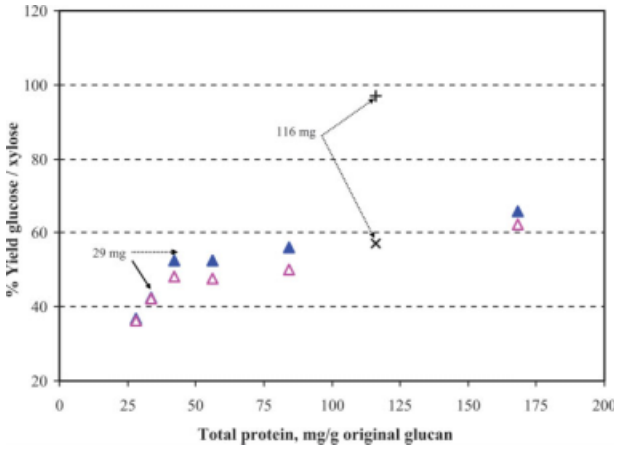

Figure 10. Glucose and xylose yields after 72 h of hydrolysis vs. total protein loadings for xylanase supplementation of constant CAB mass loadings of 29.0 mg/g original glucan for controlled pH pretreated poplar solids.

Closed symbols and × show avg. glucose yield and open symbol and "+" avg. xylose yield.

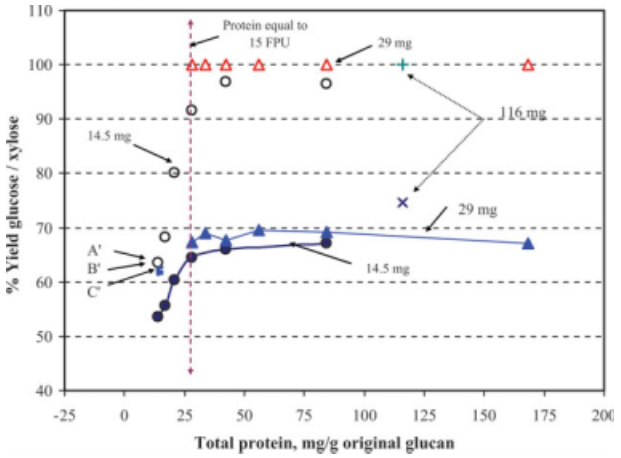

Figure 11. Glucose and xylose yields after 72 h of hydrolysis vs. total protein loadings for xylanase supplementation of constant CAB mass loadings of 29.0 and 14.5 mg/g original glucan for DA pretreated poplar solids.

Closed symbols and × show avg. glucose yield and open symbol and "+" avg. xylose yield. A′, B′, and C′ represent 14.5 mg + Tween 20, BSA, and PEG 6000, respectively, at a loading of 300 mg/g glucan (3.0 mg/mL).

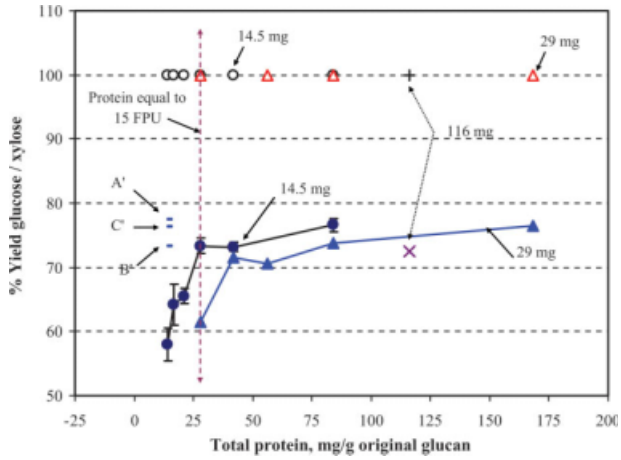

Figure 12. Glucose and xylose yields after 72 h of hydrolysis vs. total protein loadings for xylanase supplementation of constant CAB mass loadings of 29.0 and 14.5 mg/g original glucan for unwashed SO_2 pretreated poplar solids.

Closed symbols and × show avg. glucose yield and open symbol and "+" avg. xylose yield. A′, B′, and C′ represent 14.5 mg + Tween 20, BSA, and PEG 6000, respectively, at a loading of 300 mg/g glucan (3.0 mg/mL).

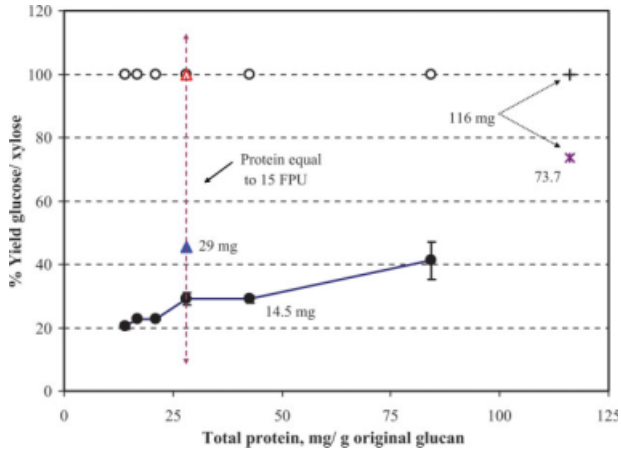

Figure 13. Glucose and xylose yields after 72 h of hydrolysis vs. total protein loadings for xylanase supplementation of constant CAB mass loadings of 14.5 mg/g original glucan for FT pretreated poplar solids.

Closed symbols and × show avg. glucose yield and open symbol and "+" avg. xylose yield.

but given the low release of sugars from the particular controlled pH poplar solids used in this study, it is expected that a further reduction in cellulase loadings will result in much lower yields than for 29.0 mg.

Low Residual Xylan Substrates

Dilute acid pretreated solids. The impact of xylanase supplementation was evaluated for poplar solids retaining lower amounts of xylan following pretreatment with dilute sulfuric acid, SO_2, and flowthrough approaches. For unwashed dilute acid pretreated poplar solids, the effect of xylanase supplementation on the release of glucose and xylose is shown in Figure 11. Compared with the more than 25% increase in glucose release when a CAB mass loading of 14.5 mg was supplemented with high levels of xylanase, xylanase supplementation provided limited improvement at a CAB mass loading of 29.0 mg. On the other hand, xylose release was essentially complete for a CAB mass loading of 29.0 mg compared with ~85% with only 14.5 mg of cellulase, and about 97% with xylanase supplementation. Additives significantly enhanced glucose yield (≥14%) when added with just cellulase for a mass loading of 14.5 mg of CAB, and from the data in Figure 11, it can be inferred that glucose release at 14.5 mg of cellulase protein supplemented with xylanase and additives will be equal to or higher than that obtained with much more cellulase alone (116.1 mg) on an equal protein basis.

Table 3. Total 72 h Glucose Yield (TGY*) and Total Xylose Yield (TXY) from the Combined Operations of Pretreatment and Enzymatic Hydrolysis At Different Protein Loadings for Poplar Solids Prepared by Leading Pretreatments

Enzyme Loading		AFEX			ARP			DA			Lime			Controlled pH			SO_2		
Protein (mg/g original glucan)	FPU (per g original glucan)	TGY	TXY	TSY	TGY	TXY	TSY	TGY	TXY	TSY	TGY	TXY	TSY	TGY	TXY	TSY	TGY	TXY	TSY
5.8	3	7.3	25.8	12.1	10.9	46.6	20.1	46.7	66.5	51.8	27.7	33.0	29.1	7.6	84.7	27.5	37.2	84.1	49.3
14.5	7.5	14.8	32.0	19.2	23.9	54.8	31.9	73.1	70.7	72.4	51.5	46.2	50.2	16.0	87.9	34.6	60.3	85.3	66.7
14.5	7.5 F + add.†	25.9	30.7	27.1	53.7	71.5	58.3	78.9	70.9	76.8	79.8	59.4	74.5	nd	nd	nd	90.8	85.3	89.4
84.2	7.5 F + MX_5	31.3	41.6	33.9	54.9	86.6	63.1	82.9	70.9	79.8	60.9	63.8	61.7	nd	nd	nd	76.7	85.3	79.0
29.0	15	29.0	41.0	32.1	44.8	65.7	50.2	83.6	70.9	80.3	63.9	56.1	61.9	22.9	88.4	39.8	70.0	85.3	73.9
56.1‡	15 F + MX_1	45.5	57.0	48.5	58.2	84.9	65.1	85.0	70.9	81.4	66.8	69.6	67.5	53.3	100.0	65.3	71.4	85.3	75.0
168.4	15 F + MX_5	61.1	72.3	64.0	63.5	94.6	71.5	85.0	70.9	81.4	68.8	72.9	69.9	65.9	105.5	76.1	71.9	85.3	75.4
116.1	60	60.0	60.5	60.1	77.0	86.9	79.6	89.8	70.9	84.9	73.1	68.6	71.9	35.7	93.9	50.7	73.8	85.3	76.8

* Definitions of TGY, TXY, and TSY are described in materials and methods section. † The highest yields obtained with an additive of three are presented, for detail see sections for each pretreatment. ‡ Total protein includes cellulase, β-glucosidase, and xylanase.

SO_2 pretreated poplar solids. SO_2 pretreated poplar solids showed an interesting trend in glucose release with xylanase supplementation. On equal protein basis, supplementation of a CAB mass loading of 14.5 mg with xylanase resulted in greater glucose release than achieved for CAB mass loadings of 29.0 or 116.1 FPU, as shown in Figure 12. In addition, additives had a significant impact on glucan digestion (≥20%) when used with just cellulase. Furthermore, it appeared that washing SO_2 pretreated poplar followed by adding xylanase and additives would give very high glucose release at a CAB protein mass loading of only 14.5 mg/g original glucan.

Flowthrough pretreated poplar solids. At a CAB mass loading of 14.5 mg, flowthrough pretreated solids showed a significant increase (>95%) in glucose release with xylanase supplementation, as observed in Figure 13. However, the yields were much higher for CAB mass loadings of 29.0 and 116.1 mg for the same mass of protein used.

Total sugar yields vs. protein

Data on the total glucose plus xylose yields following enzymatic hydrolysis of solids from different pretreatments for 72 h using different total catalytic protein mass loadings is summarized in Table 3. For all protein loadings, dilute sulfuric acid gave the highest total 72 h glucose yield, but controlled pH pretreatment of poplar gave the highest total xylose yields. The highest total sugar yield was observed with dilute acid pretreatment. Overall, yields for glucose and xylose increased with xylanase supplementation. However, for some pretreatments, the increase in glucose release was not very significant beyond low levels (MX_1, MX_2) of xylanase supplementation, especially at a CAB mass loading of 29.0 mg. Although the total sugar yield for all pretreatments increased with protein loadings, the total sugar yield per mg of total protein declined with protein loading from the highest 9.0 to 0.4, as evident from Table 3. Therefore, for economic reasons, it is necessary to identify the limit of protein supplementation.

Cellulase–xylanase interactions

Limited studies have been reported of the interactions between cellulase and xylanase,[37,48,49] in sharp contrast to the amount of attention given to the synergy among cellulase components.[50–56] However, for real applications, it is vital to understand the cooperative action between these two classes of enzymes on pretreated lignocellulosic biomass.[57] Synergism is often defined as the ratio of the rate or yield of product release by enzymes when used at the same time to the sum of rate or yield of these products when the enzymes are used separately in the same amounts as they were employed in the mixture. However, because this study was concerned with release of two products, glucose and xylose, over a range of loadings of xylanase, xylanase leverage on glucan hydrolysis was defined as the ratio of the percent increase in glucose release to the percent increase in xylose release. As shown in Figure 14 for AFEX poplar solids, a strong linear relation (R^2 = 0.97 and 0.93 for 14.5 and 29.0 mg CAB mass loading, respectively) was found between the increase in glucose release and the increase in xylose release, as determined by supplementation with multiple xylanase loadings at fixed CAB mass loadings of 14.5 and 29.0 mg. This linear relationship was observed for solids prepared by other leading pretreatments as well, inferring a strong xylanase

leverage. As shown in Figure 14a,b, the leverage ratio (LR) defined in this way decreased from 1.288 to 0.911 and 0.659 to 0.531 for AFEX and ARP solids, respectively, when the CAB mass loading was increased from 14.5 to 29.0 mg, and once again, a similar observation was made for solids prepared by the other pretreatments, as summarized in Table 4. AFEX pretreated solids showed the highest leverge ratio for cellulase–xylanase interaction at both cellulase loadings, lime and ARP poplar solids showed about 2/3 and half the leverage ratio for AFEX solids, respectively, and dilute acid showed the lowest LR. However, at a cellulase mass loading of 29.0 mg, controlled pH solids gave a higher LR than other pretreatments, even though they contain less residual xylose than AFEX, ARP, and lime pretreatments. One possible explanation for this unexpected result may be the higher amount of acetyl groups left in controlled pH pretreated solids than for other pretreatments,[58] but the effect of acetyl content on cellulase–xylanase interactions has not been established. In any event, cellulase–xylanase interactions are apparently controlled by other factors than just the amount of residual xylose. This outcome is similar to a reported observation that the rate of initial glucose release by xylanase is not dependent on the amount of residual xylose in the substrate.[36]

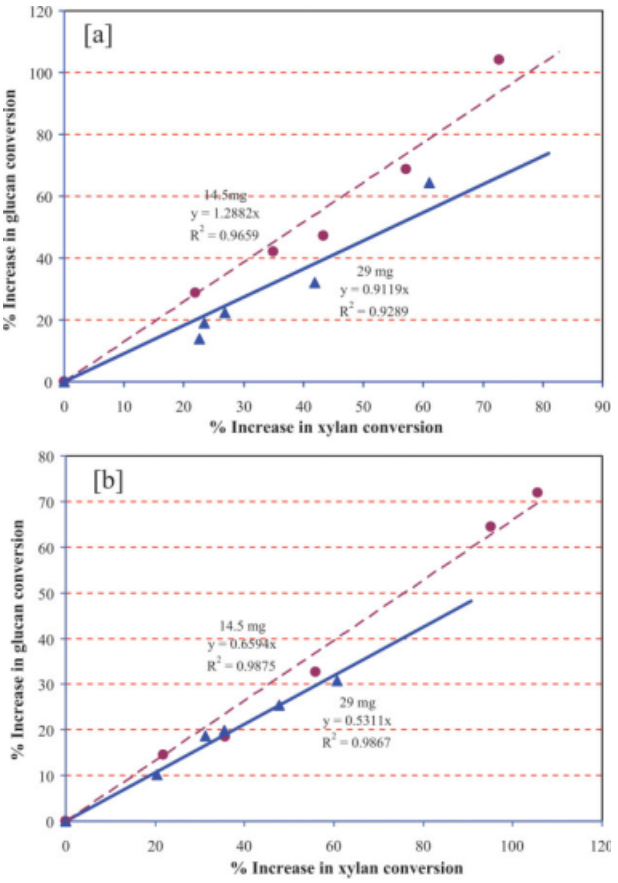

Figure 14. Relationship of enzymatic release of glucose with enzymatic removal of xylose at fixed CAB loadings of 14.5 and 29.0 mg/g original glucan with different levels of xylanase supplementations for pretreated poplar solids (a) AFEX (b) ARP.

The interactions between cellulase and xylanase were also measured in terms of the more frequently employed definition of synergy. In this case, poplar solids were hydrolyzed for 24 and 72 h at 50°C with xylanase (~28 mg/g original glucan, MX_2) and CAB (14.5 mg/g original glucan) individually and with simultaneous addition of both. The hydrolysis data, LR and synergy factors are presented in Table 4. By this definition, the synergy was again highest for AFEX pretreated poplar solids (1.35), and increased from 1.35 to 1.44 with increasing hydrolysis time from 24 to 72 h. On the other hand, for ARP pretreated solids the synergy was somewhat lower at 72 h than for 24 h (1.31 vs. 1.14) even though both ARP and AFEX use ammonia for pretreatment, albeit in different modes and amounts.

Impact of xylanase treatment on solids digestibility

Xylose removal is thought to enhance cellulose accessibility and thus result in greater release of glucose by enzymes. However, xylose removal is often performed with various chemicals at high temperatures[39,44,59] which are sure to affect more than just removal of xylose. Ghose and Bisaria reported increased glucose release for sugarcane baggase after xylanase treatment due to increased accessibility of the cellulosic regions.[48] Suurnakki et al.[60] observed increased pore size by enzymatic removal of xylose and glucomannan.

To study the effect of xylanase treatment on glucose release, poplar solids left after the different pretreatments were saccharified with just xylanase for 24 (72) h followed by addition of CAB for 72 h. The objective was to evaluate whether addition of xylanase before cellulase improves the overall digestibilility, and for that 24 and 72 h treatment times were chosen. However, due to insignificant differences in the effects of xylanase treatment for longer times, only the 24 h data was presented. These results were compared with data for experiments conducted using the same amounts of CAB and xylanase added together. Figure 15 shows the

Table 4. Leverage Ratios and Synergy Factors for Release of Glucose and Xylose at a CAB Mass Loading of 14.5 mg/g Glucan

	Glucose Released for 24 h (72 h) Hydrolysis with Cellulase and Xylanase Alone and Together				Leverage ratios (LR) (m)†, by a New Definition	
Pretreatment	Cellulase	Xylanase	Cellulase + Xylanase	Synergy Factor*	14.5 mg	29.0 mg
AFEX	14.0 (15.3)	1.4 (2.7)	20.8 (47.1)	1.35 (1.44)	1.28	0.91
ARP	23.5 (35.5)	2.9 (6.0)	34.6 (47.1)	1.31 (1.14)	0.66	0.53
DA	44.6	4.1	58.2	1.20	0.46	nd
Flowthrough	15.3	1.6	19.5	1.15	nd	nd
Lime	28.7	1.7	40.3	1.32	0.87	0.43
Controlled pH	nd	nd	nd	nd	nd	1.18
SO_2	46.2	1.6	62.0	1.30	nd	nd

* Synergy factor = (glucose yield with mixtures of cellulase + xylanase)/(glucose yield with cellulase + glucose yield with xylanase). † Leverage ratio (LR) = % increase in glucan conversion (y)/% increase in xylan conversion (x). nd, not determined.

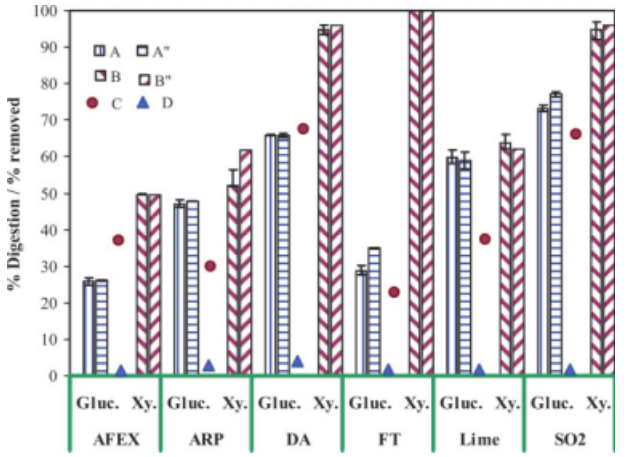

Figure 15. Impact of 24 h hydrolysis with xylanase followed by addition of cellulase on glucose and xylose release from solids resulting from pretreatment of poplar by leading technologies. A and B represent % glucose and % xylose released for cellulase and xylanase added together, A″ and B″ represent % glucose and % xylose released for hydrolysis with just xylanase followed by addition of cellulase, C represents % xylose removed from the residual left in solids, D represents % glucose removed from the residual left in solids during xylanase treatment.

extent of hydrolysis for both cases and the amount of xylose and glucose removed during xylanase treatment. Overall, there was little difference observed between the results when the substrates were treated with xylanase before cellulase addition versus when both enzymes were added together. Thus, it seems that xylanase can remove xylose either way and have the same effect on glucose release. This result contrasts the higher enhancement for simultaneous than sequential action with cellulosomal enzymes from *Clostridium cellulovorans* reported by Murashima et al.[49] Other experiments not reported here showed that the digestibilities were a little lower when cellulase was added after 72 h of xylanase treatment, possibly due to inhibition of cellulase activity by the xylooligomers and xylose generated over longer periods of xylanase treatment.[24,29,61]

Discussion

Initial comparative data were developed for enzymatic hydrolysis of poplar solids with cellulase enzymes pretreated by leading technologies. The poplar employed proved much more recalcitrant than the corn stover used before,[17,61–66] and as a result, sugar yields were much further from the theoretical maximum for all of the technologies except sulfur dioxide pretreatment even for very high protein loadings. Therefore, further refinement in pretreatment conditions was important to pursue for poplar to improve yields and also

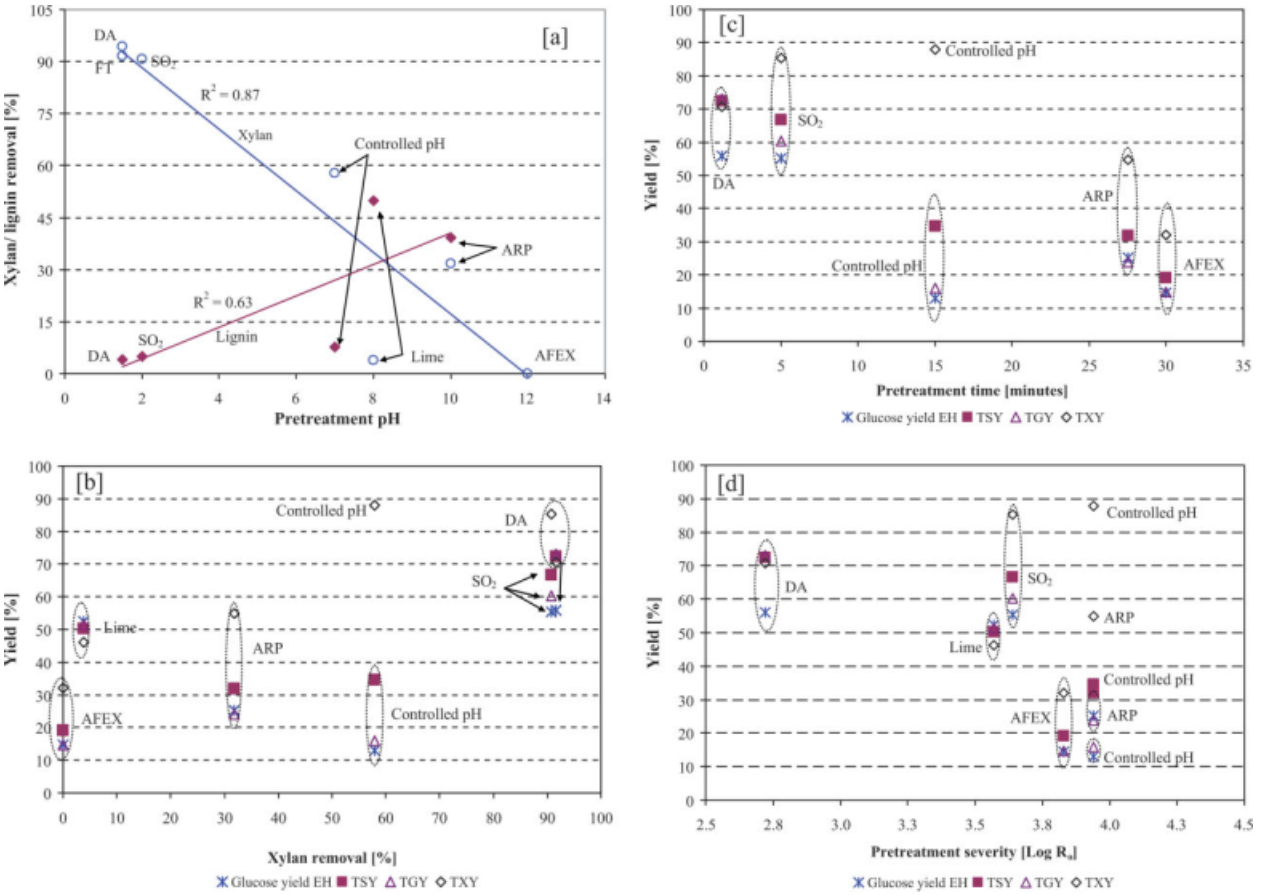

Figure 16. (a) Relationship between pretreatment pH and xylan/lignin removal, (b) the relation between xylan removal and the 72 h glucose yield^{EH}, TGY, TXY, and TSY, (c) effect of pretreatment time on 72 h glucose yield^{EH}, TGY, TXY, and TSY, and (d) effect of pretreatment severity on 72 h glucose yieldEH, TGY, TXY, and TSY. The yields taken here were for a CAB mass loading of 14.5 mg/g glucan; TGY, TXY, and TSY are defined in materials and methods section.

reduce enzyme usage, and many of the pretreatment developers employed more harsh combinations of conditions and other approaches toward this end. In addition, limited results with other sources of poplar suggest that other factors associated with the biomass material such as lignin content are important to consider in the selection of feedstocks. It is also important to note that although the focus here was on yields for times of 72 h and less to reveal trends in the rate of sugar release, enzymatic hydrolysis for longer times will likely result in higher yields that would be a better indicator of commercial potential of these pretreatment approaches.

Comparing the solids produced by the pretreatments shows that pretreatment pH influenced xylan and lignin removal more than time, temperature, or the log R_0 severity that includes these two variables, as shown in Figure 16a. In particular, more xylan was removed as the pH dropped, whereas higher pH values resulted in more lignin removal. Comparison of enzymatic glucose (yield^{EH}), total glucose (TGY), total xylose (TXY), and total sugar (TSY) yields following 72 h of enzymatic hydrolysis for the different pretreatments showed that all of these increased with xylan removal for poplar, as illustrated by Figure 16b for a CAB mass loading of 14.5 mg/g glucan. However, no obvious relation was found between these sugar yields and lignin removal except total xylose yields first increased and then dropped with lignin removal (data not shown). Sugar yields (glucose yield^{EH}, TGY, TXY, and TSY) decreased with pretreatment time and had no relation to pretreatment severity for the different pretreatments evaluated here, as shown in Figure 16c,d, respectively. Furthermore, although not shown, pretreatment temperature has no clear effect on glucose, xylose, or total sugar yields. Overall, these results would suggest that removing xylan has a greater effect on enzymatic hydrolysis yields than removing lignin; however, this conclusion does not take into account the possibility that lignin disruption could be coincidental with xylan removal,[39,67] supporting the possibility that both hemicellulose and lignin must be modified to achieve high yields in enzymatic hydrolysis.

Contrary to some hypotheses,[68] xylanase supplementation increased glucose release from the solids pretreated by all technologies, and release did not decrease with increased xylanase supplementation. The extent of enhancement varied with the pretreatment method, but because the degree of interaction between xylanase and cellulase did not correlate well with xylose content in the pretreated solids, other factors such as acetyl content may govern or at least also impact cellulase–xylanase interaction. Treatment of pretreated solids with xylanase before adding CAB did not improve glucose release, possibly due to the greater recalcitrance of poplar.

Washing pretreated solids prior to adding enzymes enhanced digestion yields considerably for all pretreatments tested, and further study is warranted on what factor is responsible for this important phenomenon. In addition, supplementation with the noncatalytic protein BSA, the soluble polymer PEG6000, and particularly the surfactant Tween 20 significantly improved sugar release by enzymes for all pretreatments despite substantial differences in their relative yields.

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