ARTICLE

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Effect of Additives on the Digestibility of Corn Stover Solids Following Pretreatment by Leading Technologies

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ABSTRACT: Bovine serum albumin (BSA), Tween-20, and polyethylene glycol (PEG6000) were added to washed corn stover solids produced by ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), dilute sulfuric acid (DA), lime, controlled pH, and sulfur dioxide (SO_2) pretreatments and to untreated corn stover (UT) and pure Avicel glucan prior to adding cellulase supplemented with β-glucosidase at an activity ratio of 1:2/g and a moderate enzyme loading of 16.1 mg/g glucan in the raw corn stover. The additives were applied individually at 150, 300, and 600 mg/g glucan in the pretreated solids and in combinations of equal amounts of each that totaled 600 mg/g. The greatest increase in total sugar release was by Tween-20 with SO_2 pretreated solids followed by PEG6000 with ARP solids and Tween-20 with lime solids. The effectiveness of the additives was observed to depend on the type of sugars left in the solids, suggesting that it may be more beneficial to use the mixture of these additives to realize a high total sugar yield. In addition, little enhancement in sugar release was possible beyond a loading of 150 mg additives/g glucan for most pretreatments, and combinations did not improve sugar release much over use of additives alone for all except SO_2. Additives were also found to significantly increase concentrations of cellobiose and cellooligomers after 72 h of Avicel hydrolysis.

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KEYWORDS: corn stover; pretreatment; enzymatic hydrolysis; cellulase; β-glucosidase; additives; BSA; Tween-20; PEG6000

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Introduction

Acids (Bienkowski et al., 1984; Ghosh and Ghose, 2003; Goldstein et al., 1989; Lynd, 1996; Sivers and Zacchi, 1996) or active proteins (Lynd et al., 2008; Wyman et al., 2005a) can breakdown cellulose and hemicellulose in cellulosic biomass to sugar oligomers and monomers for fermentation to ethanol or other products. However, although the biological route to sugar release from cellulosic biomass offers high yields, the quantity of enzymes needed for conversion with high yields is high and remains among the primary impediments to cellulosic ethanol commercialization (Wyman, 2007; Yang and Wyman, 2008b). In a recent study, pretreatments by ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), controlled pH, dilute acid, and lime realized similarly high glucose and xylose yields from corn stover for the combined operations of pretreatment and enzymatic hydrolysis at cellulase mass loadings of about 55 and 220 mg of protein/g glucan in the original corn stover (corresponding to about 15 and 60 FPU respectively) (Mosier et al., 2005; Wyman et al., 2005a). However, even the lowest level corresponds to about 0.25 lbs of enzyme/gal ethanol, and because enzymes are so expensive (Howard et al., 2003; Wingren et al., 2005; Wyman, 2007), high sugar yields must be achieved with much lower protein usage (Merino Sandra and Cherry, 2007; Wyman, 2007). It is believed that non-productive binding of cellulase and other enzymes to lignin and other portions of the solid (Excoffier et al., 1991; Yang and Wyman, 2006); enzyme deactivation over time due to prolonged exposure to shear, mixing, and temperature (Desai and Converse, 1997; Gunjikar et al., 2001; Kaya et al., 1996; Kim et al., 1982; Vlasenko et al., 1997; Wang et al., 2006); and deactivation by sugar and lignin and their degradation products (Garcia-Aparicio et al., 2006; Kaya

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et al., 1999; Kumar and Wyman, 2008d,e; Mes-Hartree and Saddler, 1983; Panagiotou and Olsson, 2007; Sineiro et al., 1997) are at least partly responsible for high enzyme loading requirements. But the complexity of the substrate and the enzymes has confounded developing a clear picture of the mechanism.

Although more effective enzymes with increased specific activity (Kumar and Wyman, 2008b,c; Merino Sandra and Cherry, 2007; Percival Zhang et al., 2006), better pretreatments (Kadam and Hsu, 1997; Wyman et al., 2005b), and improved hydrolysis/fermentation systems and conditions should reduce the enzyme requirement for cellulosic biomass saccharification (Lynd et al., 2005; Oehgren et al., 2007; Tu et al., 2007b; Wingren et al., 2003), additives such as non-catalytic proteins (Willies, 2007; Xu et al., 2008; Yang and Wyman, 2006; Zheng et al., 2008), surfactants (Alkasrawi et al., 2003; Helle et al., 1993; Kim et al., 1982, 2007; Ooshima et al., 1986; Park et al., 1992; Wu and Ju, 1998), polymers (Boerjesson et al., 2007; Borjesson et al., 2007; Tjerneld et al., 1985), and polyelectrolytes (Feng et al., 1992) have been shown to significantly increase sugar yields and/or lower enzyme requirements to achieve a given yield (Alkasrawi et al., 2003; Castanon and Wilke, 1981; Yang and Wyman, 2006). The mechanism is still not entirely clear, but such additives have been thought to impede deactivation and/or unproductive binding, increase cellulose accessibility, and/or enhance enzyme activity (Eriksson et al., 2002; Huang et al., 2003; Kim et al., 2006; Mizutani et al., 2002; Sewalt et al., 1997; Yang and Wyman, 2006).

Bovine serum albumin (BSA) actually has long been used in biotechnology research as a blocking agent (Huang et al., 2003) to protect proteins of interest from non-specific adsorption to walls of glassware and other equipment (Ha et al., 1993; Palonen, 2004), and due to its higher hydrophobicity and strong interaction with surfaces, it may displace other proteins. BSA probably has a great affinity for lignin and lignin containing substrates due to its highly hydrophobic nature, isoelectric point (p*I*), and formation of highly hydrophobic agglomerates at temperatures above about 50°C (Echterhoff et al., 2001) and could coat exposed lignin to prevent unspecific, unproductive cellulase adsorption, thus making more enzyme available for hydrolysis (Willies, 2007; Yang and Wyman, 2006). Furthermore, BSA may enhance enzyme stability (Huang and Monk, 2004; Robert, 1983) and reduce the hydrophobicity of surfaces, facilitating cellulase adsorption and desorption (Niamsiri et al., 2007; Park et al., 2002; Tilton et al., 1991). However, the interaction of BSA with the substrate surface may be affected by temperature, pH, ionic strength, substrate hydrophobicity, and surface charge (Chandra et al., 2007; Echterhoff et al., 2001; Ha et al., 1993; Halder et al., 2005).

Non-ionic surfactants and polymers have also shown promise for improving enzymatic hydrolysis or reducing enzyme demands, perhaps by increasing cellulase activity and stability, enhancing cellulose accessibility, and/or decreasing surface tension (Boerjesson et al., 2007; Kamande et al., 2000; Kim et al., 1982; Kristensen et al., 2007; Park et al., 1992; Tu et al., 2007a,b; Wu and Ju, 1998). Surfactants and polymers are hydrophobic and believed to form a coating on the hydrophobic lignin surface, reducing irreversible protein adsorption that could lead to deactivation. Another plausible mechanism is that cellulase entrapment in reverse micelles formed by the surfactant reduces detrimental effects of heat and solvents on enzyme activity (Chen et al., 2006; Xiang et al., 2006). Addition of surfactants during pretreatment facilitates removal of lignin and its degradation products and enhances sugar yields by increasing cellulose accessibility (Kurakake et al., 1994), with their effectiveness depending on the type of surfactant and substrate (Kim et al., 2007). Surfactants have been reported to enhance digestibility of pure cellulose (Helle et al., 1993; Ooshima et al., 1986) as well, with the cause attributed to enhanced stability and increased productive and/or decreased unproductive adsorption of cellulase components (Kim et al., 1997; Tjerneld et al., 1985). Furthermore, their effectiveness is seemingly affected by substrate structural properties, for example, crystallinity (Gama and Mota, 1997; Ooshima et al., 1986).

Additives are reported to have a pronounced effect on solids produced by dilute acid and steam explosion pretreatments (Kristensen et al., 2007; Pan et al., 2005; Yang and Wyman, 2006), but surfactants were reported to also improve performance with solids from lime pretreatment (Kaar and Holtzapple, 1998; Tu et al., 2007b). However, limited information has been reported on the use of additives with a range of pretreatments, and no comparative information is available on the effects of additives on enzymatic digestion of solids prepared by a range of promising pretreatments. In addition, the impact of additive loadings on results for these pretreatment has not been reported. Therefore in this study, we sought to determine how a leading non-catalytic protein (BSA), surfactant (Tween-20), and polymer (PEG6000) affect glucose and xylose release after 72 h of enzymatic hydrolysis of corn stover solids resulting from pretreatment using AFEX, ARP, dilute sulfuric acid (DA), lime, controlled pH, and sulfur dioxide (SO_2) technologies as well as pure Avicel glucan and untreated corn stover. Data on the effect of acid soluble lignin on the effectiveness of additives is also presented.

Materials and Methods

Materials

Pure cellulose, Avicel PH-101, was purchased from FMC Corporation (Philadelphia, PA) (Cat 11365, Lot 1094627); 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). Unpretreated Kramer corn stover was generously provided by the National Renewable

Energy Laboratory (NREL) in Golden, CO. Solids resulting from corn stover pretreatment by various technologies were generously provided by our partners in the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI): ARP by Auburn University, AFEX by Michigan State University, dilute acid pretreatment with the Sunds pilot reactor by NREL, controlled pH by Purdue University, lime by Texas A&M University, and sulfur dioxide by the University of British Columbia. Dr. Michael Studer and Dr. Simone Brethauer at the University of California, Riverside provided water washed dilute acid pretreated solids using a Parr reactor. ARP, lime, and dilute acid pretreated corn stover solids were received already washed, and AFEX and SO_2 solids were washed with DI water (WW) in three steps with the total water volume equal to 30 times the wet weight of the biomass. Controlled pH pretreated corn stover solids that had been washed with hot water were generously provided by Purdue University. The reaction conditions and washed solids compositions as determined according to NREL Laboratory Analytical Procedure 002 (NREL, 2004) are reported in Table I for all of the pretreatments. Acid soluble lignin was prepared by dilute acid pretreatment of a 5% (w/w) solids loading of corn stover at 140°C with 1.0% (w/w) sulfuric acid for 40 min in our 1 L Parr reactor. The pretreatment liquor was neutralized with $CaCO_3$ and found to contain 1.0, 10.0, and 2 g/L of soluble lignin, xylose, and glucose, respectively, as determined by NREL Laboratory Analytical Procedure 002 (NREL, 2004).

Enzymes

Spezyme® CP cellulase (lot 301-04075-034; 59 ± 5 FPU/mL, 123 ± 10 mg protein/mL), GC® 220 cellulase (lot 301-04232-162; 90 ± 5 FPU/mL, 184 ± 10 mg protein/mL), Multifect® Xylanase (lot 301-04021-015; 42 ± 5 mg protein/mL), and β-glucosidase (31 ± 5 mg protein/mL) enzymes were generously provided by the Genencor Division of Danisco US, Inc. (Rochester, NY). β-glucosidase (Novozyme188, 140 ± 5 mg protein/mL; 665 CBU/mL) used in some experiments was purchased from Sigma Chemicals.

Enzymatic Hydrolysis

Enzymatic hydrolysis was performed according to NREL Laboratory Analytical Procedure LAP 009 in at least duplicates at 1% (w/v) glucan concentrations in 0.05 M citrate buffer (pH = ~4.8) containing antibiotics (400 μL/100 mL of 10 mg/mL tetracycline in 70% ethanol and 300 μL/100 mL of 10 mg/mL cyclohexamide in DI water). These ingredients were mixed in 125 mL Erlenmeyer flasks and controlled at 48 ± 3°C using a thermostated shaker water bath set at ~200 rpm (NREL, 1996). Substrate blanks without enzyme and enzyme blanks without substrate were run in parallel. Digestibility was determined at cellulase plus β-glucosidase loadings of 16.1 mg of protein/g glucan in the raw biomass (corresponding to about 7.5 FPU/g original glucan) supplemented with β-glucosidase at a CBU to FPU activity ratio of ~2, unless otherwise stated. To evaluate the effect of additives, solids containing 1% (w/v) glucan were incubated with BSA, Tween-20, or PEG6000 for at least 4 h prior to enzyme addition (Yang and Wyman, 2006). Three different additives loadings of 150, 300, or 600 mg/g glucan in pretreated solids were used, and equal amounts of additives totaling 600 mg/g glucan were applied at either 300 mg/g glucan each for 2 of the 3 or 200 mg/g glucan each for all three. Samples of about 700 μL in volume drawn at 24, 48, and 72 h were filtered through a 0.2 μm nylon filter vials (Alltech Associates, Inc., Deerfield, IL), and a small amount (~30 mg) of AG50W-X8 resin (Bio-Rad Laboratories, 2000 Alfred Nobel Dr., Hercules, CA, Cat 143-5441) was added to the samples having surfactant or polymer to minimize damage to the HPLC columns and especially the Aminex HPX-87P column. For resin to adsorb polymer or surfactant, filter vials with samples were placed in the refrigerator (4°C) for at least 4 h prior to filtration. Then samples were filtered, pipetted into 500 μL polyethylene HPLC vials (Alltech Associates, Inc.), and kept refrigerated at 4°C or frozen at −20°C for longer times until analyzed. 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).

Table I. Pretreatment methods, conditions, percent of glucan and xylan left in solids, and solids compositions for solids prepared by leading technologies.

		Percent of original left in pretreated solids (%)		Composition of pretreated solids (%)		
Pretreatment	Pretreatment conditions	Glucan	Xylan	Glucan	Xylan	Lignin
AFEX	90°C, 220 psi, 1:1 NH_3 to Biomass, 5 min-W	100.0	100.0	34.4	22.8	18.0
ARP	170°C, 325 psi, 3.33:1 NH_3:Dry Wt, 20 min, and 3.3 mL/g of corn stover-W	98.6	48.1	61.9	17.9	8.8
Dilute acid	Sunds System 180°C, 0.03H_2SO_4:Dry wt, 90 s, 25% solids-HW	93.4	27.2	59.3	9.3	22.5
	Parr Reactor 140°C, 0.01H_2SO_4:Dry wt, 40 min, 5% solids-W	NA	NA	53.5	3.97	22.5
Lime	55°C, 0.5:1 $Ca(OH)_2$ to Biomass (dry wt), 4 weeks, water loading—10 g/g dry biomass-W_1	97.1	NA	56.7	26.4	14.6
Controlled pH	190°C, 15 min (+5 min heat up)-HW	94.1	NA	52.7	16.2	25.2
SO_2	190°C, 5 min, 3% SO_2—steam explosion-W	96.9	NA	56.9	11.6	23.8

W, water washed; HW, hot water washed; W1, neutralized and washed; NA, not available.

Determination of Oligomers

After 72 h of enzymatic hydrolysis, the contents were centrifuged to separate solids (undigested cellulose and hemicellulose and insoluble lignin) from the liquid. Then, the solid free liquid was incubated for 1 h with 4% sulfuric acid at 121°C in an autoclave along with sugar recovery standards, as described elsewhere (Yang and Wyman, 2008a). Thereafter, the liquid was neutralized with $CaCO_3$, and the total amount of glucose and xylose analyzed using the HPLC.

The percent yield of cellooligomers with a degree of polymerization > cellobiose, G_{3+}, was calculated at follows:

$$G_{3+} = 100 \times \frac{(\text{glucose after post-hydrolysis} - \text{glucose before post-hydrolysis} - 1.053 \times \text{cellobiose before post-hydrolysis})}{\text{total potential glucose in pretreated solids}}$$

X_{2+}, the percent yield of xylooligomers containing two or more xylose units, was calculated as:

$$X_{2+} = 100 \times \frac{(\text{xylose after post-hydrolysis} - \text{xylose before post-hydrolysis})}{\text{total potential xylose in pretreated solids}}$$

in which yields after post-hydrolysis were corrected for sugar degradation using a sugar recovery standard (Yang and Wyman, 2008a).

Percentage Increase of Sugar Yield

The maximum percentage increase possible for glucose, xylose, and total sugar yield and their actual percentage increase were calculated as follows:

$$\text{Maximum possible percentage increase in yield} = 100 \times \left[\frac{M-C}{C}\right]$$
$$\text{Percentage increase in sugar yield} = 100 \times \left[\frac{X-C}{C}\right]$$
$$\text{Total sugar yield} = \text{glucose yield} + \text{xylose yield}$$

in which M is the maximum theoretical sugar yield (100%), C is the percentage sugar yield of control, and X is the percentage sugar yield with additives or at a higher enzyme loading than for the control.

Acid Soluble Lignin Effect on Avicel Hydrolysis

To determine the impact of soluble lignin on cellulase effectiveness and whether any resulting inhibition could be overcome by additives, about 0.9 g/L of soluble lignin was added to a 1% (w/v) concentration of Avicel glucan at a cellulase plus β-glucosidase loading of about 32.2 mg/g glucan (15 FPU) and hydrolyzed for 2 h. Inhibition by the sugars in the acid soluble lignin solution was measured in a parallel set of hydrolysis experiments run with addition of the same amounts of just the sugars (~10 and 2 g/L of xylose and glucose, respectively). To understand the interaction of additives with soluble lignin, cellulose alone and cellulose mixed with soluble lignin were incubated with BSA, Tween-20, or PEG6000 at hydrolysis conditions and a loading of 300 mg/g glucan for 4 h prior to enzyme addition.

Results

Impact of Additives

Avicel

Additives enhanced release of glucose from Avicel by up to 64%, as shown in Figure 1. These results contrast with those of Eriksson et al. (2002) in which surfactants only enhanced the digestibility of lignin containing substrates for enzyme loadings of ~27.6 FPU/g glucan . Similarly, Yang and Wyman (2006) reported a negligible effect of BSA on glucose release from Avicel at a cellulase loading of 15 FPU/g glucan. Thus, the results here with lower enzyme loadings suggest that additives may also improve yields by enhancing the

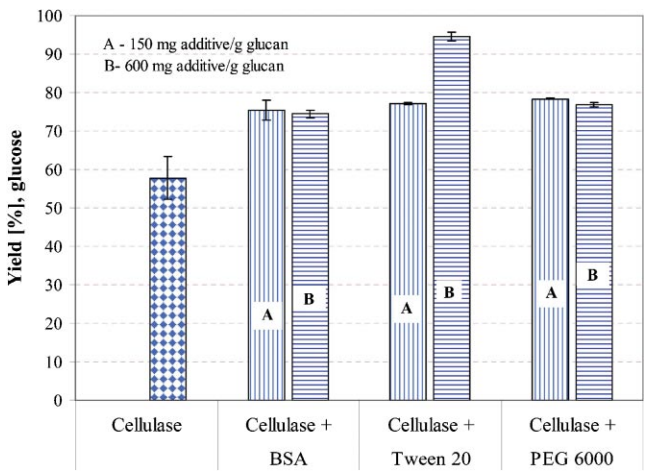

Figure 1. Impact of using 150 (labeled A) and 600 (labeled B) mg of additives/g glucan on 72 h glucose yields for enzymatic hydrolysis of Avicel at a combined cellulase plus β-glucosidase mass loading of 16.1 mg/g glucan compared to the control without additive addition on the left. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

stability, availability, and/or activity of enzymes (Kaar and Holtzapple, 1998) in addition to reducing non-productive binding of cellulase to lignin. Figure 1 also shows that glucose release continued to increase with Tween-20 loading but not appreciably with addition of more BSA and PEG6000 (74–78%), suggesting that cellulase and probably substrate loading could affect the effectiveness of additives.

Untreated Corn Stover

Because no studies were found that considered the effect of additives on untreated biomass, raw corn stover was digested with a cellulase plus β-glucosidase mass loading of 16.1 mg/g glucan, as a control, and additives loadings of 150, 300, or 600 mg/g glucan (0.15%, 0.30%, or 0.6%). As shown in Figure 2, additives enhanced glucose and xylose release modestly, with the upper limits being about 20% and 36%, respectively, with BSA. However, sugar yields were still much lower than for the pure cellulose results presented earlier or those for any of the pretreatments that follow.

Sulfur Dioxide Pretreated Solids

BSA and Tween-20 enhanced 72 h glucose and xylose release by about 50–65% and 20–70%, respectively, from solids that were pretreated with sulfur dioxide and then were DI water washed, respectively, as shown in Figure 3. However, Tween-20 required higher loadings to realize a similar effect as BSA, and PEG6000 had a much lower effect on glucose release than either of the other two (∼10%). Consistent with these results but with Douglas fir, Yang and Wyman reported about a 35% increase in glucose release by adding 1% BSA before 20 FPU/g glucan of cellulase to solids that had been pretreated with SO_2 catalyzed steam explosion (Yang and Wyman, 2006). Figure 3 also shows that mixtures of additives enhanced glucose and xylose release more than when they were used individually, suggesting that, for a given pretreatment, they may not all act by the same mechanisms. Similar to other pretreatments, xylan digestion was incomplete, probably due to the low xylanase and β-xylosidase activity of Spezyme CP.

Dilute Acid Pretreated Solids

Data were developed for glucose and xylose release by enzymatic digestion of water washed solids produced by dilute acid pretreatment of corn stover in the NREL Sunds reactor and in the Parr reactor at University of California Riverside at an enzyme mass loading of 16.1 and 6.2 mg, respectively, of cellulase plus β-glucosidase/g of glucan for 72 h with and without additives, as shown in Figure 4a and b. Because the solids from the Parr reactor were almost completely digestible at an enzyme loading of 16.1 mg (data not shown), a lower mass loading of cellulase plus β-glucosidase of 6.2 mg/glucan (about 3.0 FPU) was used to study the additives effect. For dilute acid solids from the Sunds system, as before, three different additives were used at three different loadings and in combination with equal amounts of each, however for solids with Parr reactor only the highest loading of 600 mg additives/g glucan was used. Additives had a negligible effect on glucose (<6%) and xylose (∼10%) release for the Sunds solids, as shown in

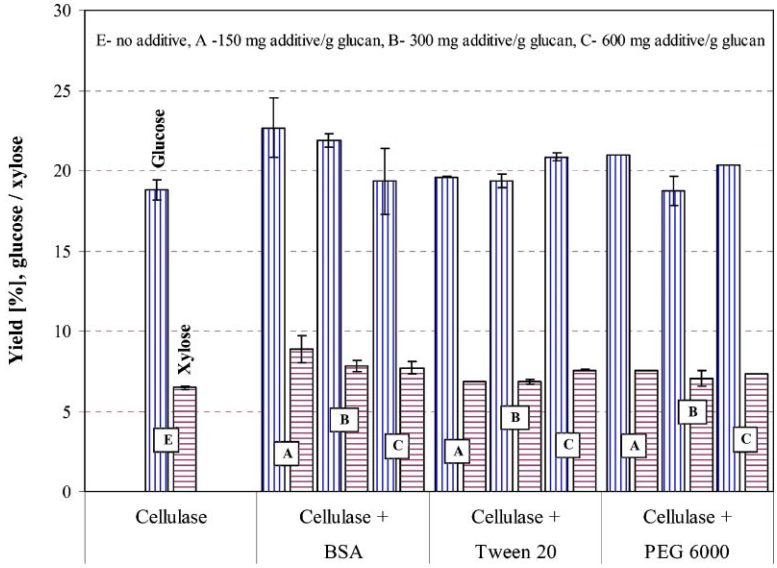

Figure 2. Impact of applying 150 (A), 300 (B), and 600 (C) mg of BSA, Tween-20, or PEG6000/g glucan on 72 h glucose and xylose release from raw corn stover at a combined cellulase plus β-glucosidase mass loading of 16.1 mg/g glucan. The results for the control with just addition of enzymes are shown on the left. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

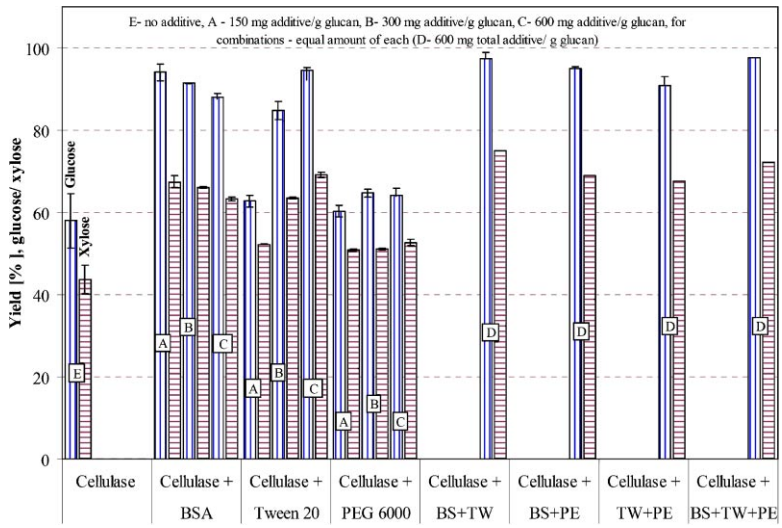

Figure 3. Impact of applying 150 (A), 300 (B), or 600 (C) mg of BSA, Tween-20, or PEG6000 additives/g glucan on 72 h glucose and xylose release from water washed SO_2 pretreated corn stover at a combined cellulase plus β-glucosidase mass loading of 16.1 mg/ g original glucan. Also shown are results for combinations of equal amounts of each of these additives at a total loading of 600 mg/glucan (D) and for the control (E) with just enzyme added. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 4a, a finding that seems to be inconsistent with the pronounced positive effect of additives reported in the literature for dilute acid and steam explosion pretreatments (Kristensen et al., 2007; Yang and Wyman, 2006). However, glucose yields were also much lower for this substrate than typical for dilute acid or uncatalyzed steam explosion, and limited accessibility to enzymes probably reduced the effectiveness of additives in a similar manner to that seen for our data with unpretreated corn stover. In addition, the affinity of lignin for cellulase and additives and the hindrance of enzymes by lignin have been shown to be affected by pretreatment conditions (Ooshima et al., 1990). Figure 4b shows results for the Parr reactor solids for an enzyme mass loading of 6.2 mg, with additives increasing glucose release by about 36% and xylose release by about 18.4% to virtually complete conversion for both. In addition, Tween-20 and PEG6000 were somewhat more effective than BSA for dilute acid pretreated solids from the Parr reactor.

Controlled pH Pretreated Solids

The same additive loadings and combinations were also applied to solids that had been pretreated with controlled pH technology and washed with hot water prior to adding the same low doses of cellulase. In this case, glucose release improved by 5–13% and xylose release by 5–20%, as shown in Figure 5. BSA proved more effective than Tween-20 or PEG6000 at these lower enzyme loadings, and sugar release did not increase with additives concentration. Unlike sulfur dioxide pretreated solids, using these additives in combination provided no benefit over employing them alone, suggesting that additives do not play different roles in enzymatic hydrolysis of this substrate. Others have reported this absence of synergy among these additives but found that mixtures of non-ionic surfactants enhanced performance more than when taken individually (Eriksson et al., 2002).

AFEX Pretreated Solids

The effect of additives on 72 h glucose and xylose release for washed AFEX pretreated corn stover solids is shown in Figure 6. Compared to pure cellulose, additives moderately enhanced (up to 14%) glucose and xylose release at a cellulase plus β-glucosidase mass loading of 16.1 mg/g glucan, somewhat better than the 7.7% enhancement reported in the literature with a much higher BSA concentration of 1% and a higher cellulase loading of 15 FPU (Yang and Wyman, 2006). Thus, cellulase loading may affect the effectiveness of additives. Figure 6 also shows that additives concentration did not affect glucose or xylose release significantly and that combinations of additives did not increase sugar release beyond results when the same additives were added individually, again different than for sulfur dioxide but similar to dilute acid and controlled pH.

ARP Pretreated Solids

At the same cellulase plus β-glucosidase mass loading of 16.1 mg/g glucan, additives had a major impact on both glucose and xylose release from ARP pretreated solids that had been washed, as shown in Figure 7. In this case, additives

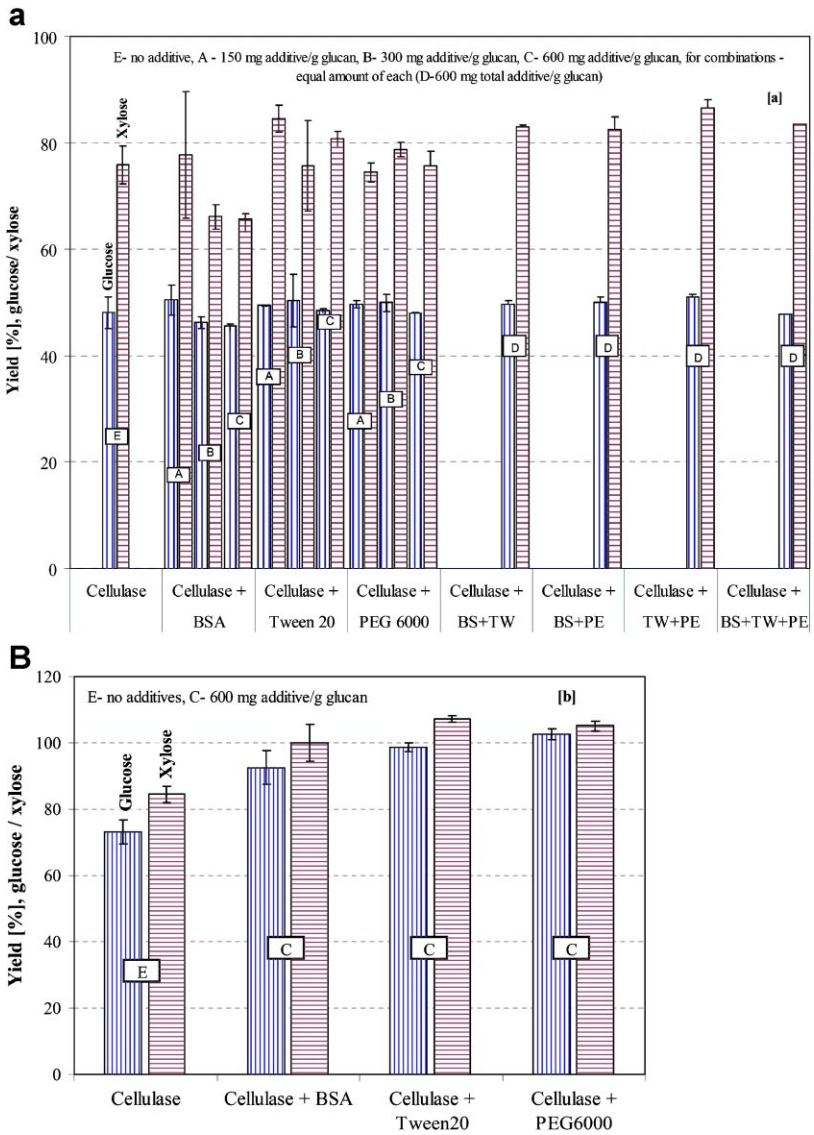

Figure 4. Impact of applying 150 (A), 300 (B), or 600 (C) mg of BSA, Tween-20, or PEG6000 additives/g glucan on 72 h glucose and xylose release at a combined cellulase plus β-glucosidase mass loading of 16.1 mg/g original glucan for (**a**) hot water washed dilute acid pretreated corn stover from the Sunds reactor and (**b**) at a cellulase together with β-glucosidase mass loading of 6.2 mg/g original glucan and 600 mg of additives/g glucan for water washed dilute acid pretreated corn stover from the Parr reactor. Also shown are results for dilute acid pretreated corn stover from the Sunds reactor employing combinations of equal amounts of each additive at a total loading of 600 mg/glucan (D) and for the control (E) with just enzyme added. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

increased glucose yields by about 50–55%, resulting in nearly complete glucose removal from the pretreated solids. However, although the increase in xylose yield (∼50%) was comparable to that for glucose, some xylan was still left in the solids or as dissolved higher oligomers, probably due to the low activity of xylanase and β-xylosidase in Spezyme CP, as discussed above (Dien et al., 2008; Kumar and Wyman, 2008a). In addition, glucose and xylose release with additives at a cellulase plus β-glucosidase mass loading of 16.1 mg/g glucan was comparable to that for a much higher cellulase plus β-glucosidase mass loading of 129 mg/g original glucan (data not shown). Furthermore, sugar yields did not increase much with additives concentration, possibly because higher ratios of additives to lignin resulted from the low lignin content of only 8% on a dry basis for the ARP solids used in this study.

Lime Pretreated Solids

For lime pretreatment, Tween-20 enhanced glucose and xylose release much more (∼20% and 74%, respectively) than BSA (∼3% and 25%, respectively), as shown in Figure 8. Furthermore, sugar release increased with concentration for Tween-20, consistent with results reported by Kaar and Holtzapple (1998). The limited

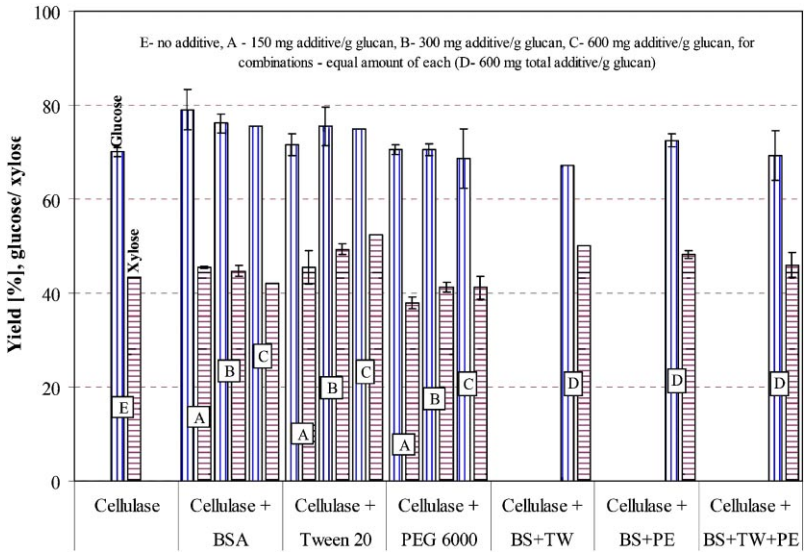

Figure 5. Impact of applying 150 (A), 300 (B), or 600 (C) mg of BSA, Tween-20, or PEG6000 additives/g glucan on 72 h glucose and xylose release from hot water washed controlled pH pretreated corn stover at a combined cellulase plus β-glucosidase mass loading of 16.1 mg/ g original glucan. Also shown are results for combinations of equal amounts of each of these additives at a total loading of 600 mg/glucan (D) and for the control (E) with just enzyme added. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

impact of BSA could be due to the relationship of its isoelectric point (p*I*, in water at 25°C = ~4.7) to the surface acidity/basicity of the solids following lime pretreatment (Echterhoff et al., 2001; Halder et al., 2005). Furthermore, the higher hydrophobicity of lime pretreated solids compared to all but ARP pretreatment may affect BSA adsorption (Chandra et al., 2007; Eriksson et al., 2002) (Kumar and Wyman, unpublished work).

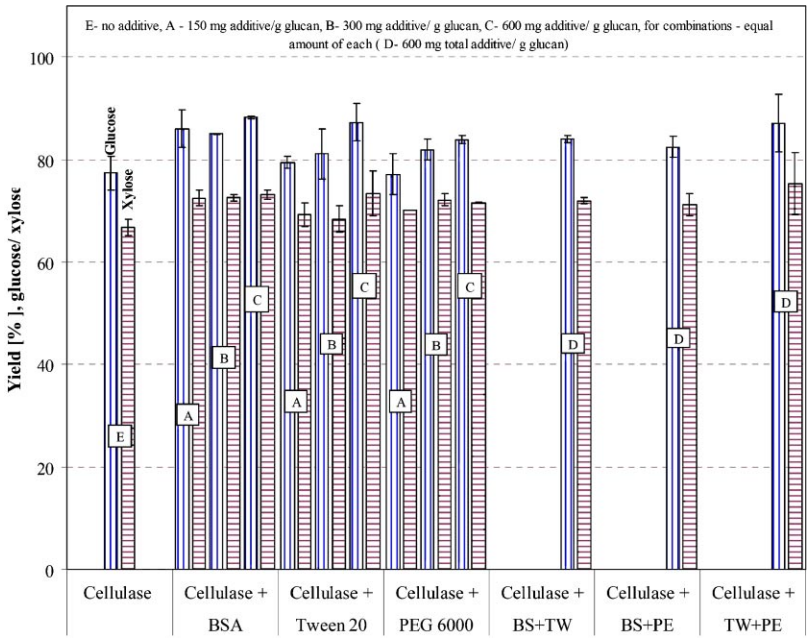

Figure 6. Impact of applying 150 (A), 300 (B), or 600 (C) mg of BSA, Tween-20, or PEG6000 additives/g glucan on 72 h glucose and xylose release from washed AFEX pretreated corn stover at a combined cellulase plus β-glucosidase mass loading of 16.1 mg/g original glucan. Also shown are results for combinations of equal amounts of each of these additives at a total loading of 600 mg/glucan (D) and for the control (E) with just enzyme added. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

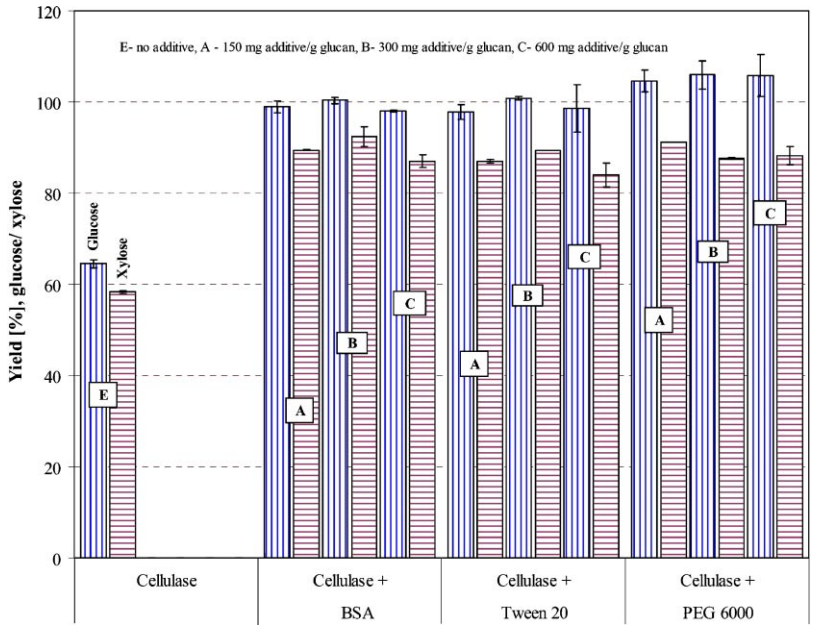

Figure 7. Impact of applying 150 (A), 300 (B), or 600 (C) mg of BSA, Tween-20, or PEG6000 additives/g glucan on 72 h glucose and xylose release from water washed ARP pretreated corn stover at a combined cellulase plus β-glucosidase mass loading of 16.1 mg/g original glucan. Also shown are results for the control (E) with just enzyme added. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Effect of Hydrolysis Time

As discussed earlier, cellulase loading apparently affected the impact of additives adversely. However, as shown in Figure 9a and b for Avicel and corn stover solids resulting from DA pretreatment with the Parr reactor, respectively, the duration of hydrolysis significantly enhanced the effectiveness of additives at a cellulase plus β-glucosidase mass loading of 16.1 and 6.2 mg/g glucan, respectively. In the case of Avicel hydrolysis, glucose yields increased from

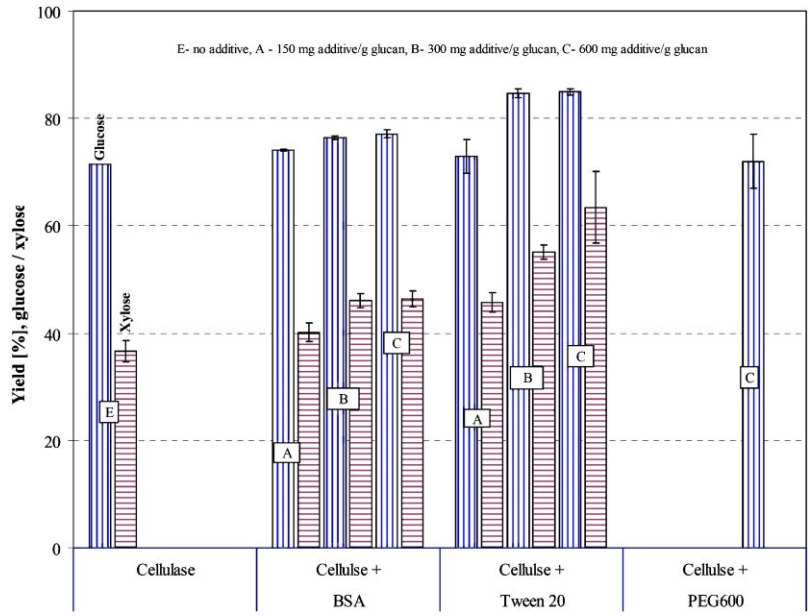

Figure 8. Impact of applying 150 (A), 300 (B), or 600 (C) mg of BSA, Tween-20, or PEG6000 additives/g glucan on 72 h glucose and xylose release from water washed and neutralized lime pretreated corn stover at a combined cellulase plus β-glucosidase mass loading of 16.1 mg/ g original glucan. Also shown are results for the control (E) with just enzyme added. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

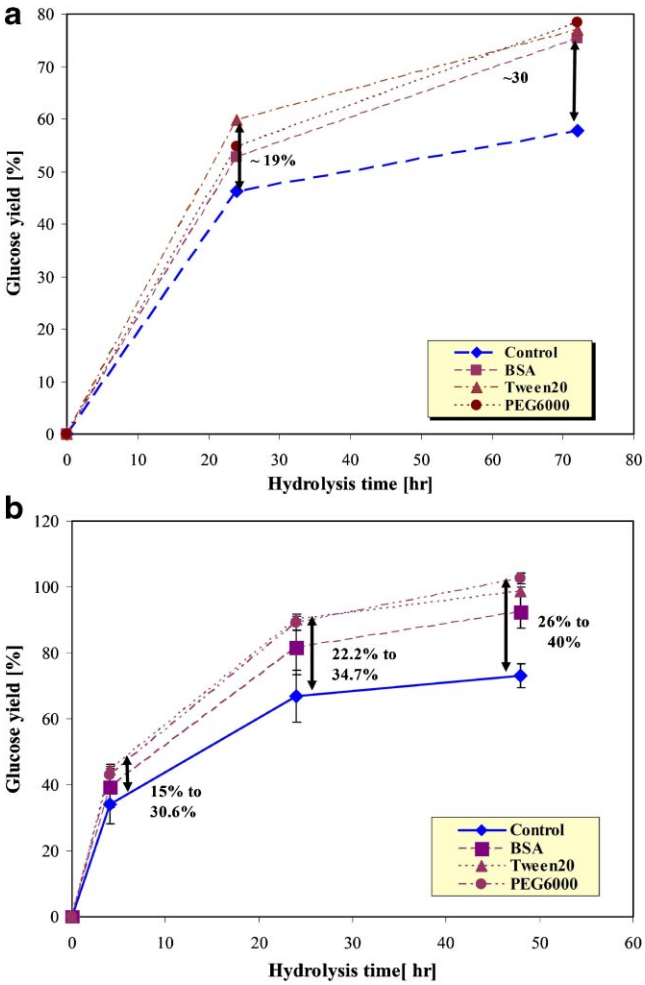

Figure 9. Glucose yields over time for enzymatic hydrolysis with (**a**) 150 mg of additives/g glucan Avicel at a combined cellulase plus β-glucosidase mass loading of 16.1 mg/g glucan and (**b**) 600 mg additives/g glucan with a combined cellulase plus β-glucosidase mass loading of 6.2 mg/g glucan applied to dilute acid pretreated corn stover from the Parr reactor. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

only about 14–19% after 24 h to ~30% after 72 h of hydrolysis, while for dilute acid pretreated corn stover, glucose yields increased from about 22–35% after 24 h to 26–40% after 48 h, depending on the additive type. Thus, it appears that additives could reduce loss of enzyme activity, perhaps by reducing the effects of prolonged exposure to air, heat, and/or agitation.

The Effect of Additives on Cellulooligomers From Avicel

The liquid resulting after 72 h of enzymatic hydrolysis of Avicel was analyzed for cellobiose and longer chain length cellooligomers (>cellobiose), as described in the Materials and Methods Section. Data for Avicel hydrolysis at a cellulase plus β-glucosidase mass loading of 16.1 mg/g glucan with 600 mg additives/g glucan is shown in Figure 10. In this case, cellobiose yields increased by about 68–74% and higher cellooligomers ($>G_2$) by about 44–110%. Thus, additives seemed to either accelerate the action of CBH and/or reduced inhibition of cellulase by oligomers, as observed elsewhere (Kumar and Wyman, 2008a).

Effect of Soluble Lignin

Based on results by Berlin et al. (2006) that soluble lignin components may inhibit enzyme activity, the effect of adding soluble lignin prior to Avicel hydrolysis was studied. Although additives improved hydrolysis, acid soluble lignin and the accompanying sugars repressed the initial hydrolysis rate significantly, as shown by comparing the cellulase only results on the left of Figure 11 to those with the additives and soluble lignin to the right. Although additives relieved inhibition by acid soluble lignin considerably, the enhancement in glucose release was not comparable to the results without lignin added. However, the fact that adding just sugars in an amount equal to that present with the acid soluble lignin gave the same inhibition as the acid soluble lignin solution indicates that the sugars in the acid soluble lignin mixture were primarily responsible for cellulase inhibition. This suggests that the lignin itself had a limited effect, if any.

Summary of Additives Impact on Sugar Release

The additives BSA, Tween-20, and PEG6000 enhanced sugar yields from solids prepared by leading pretreatment

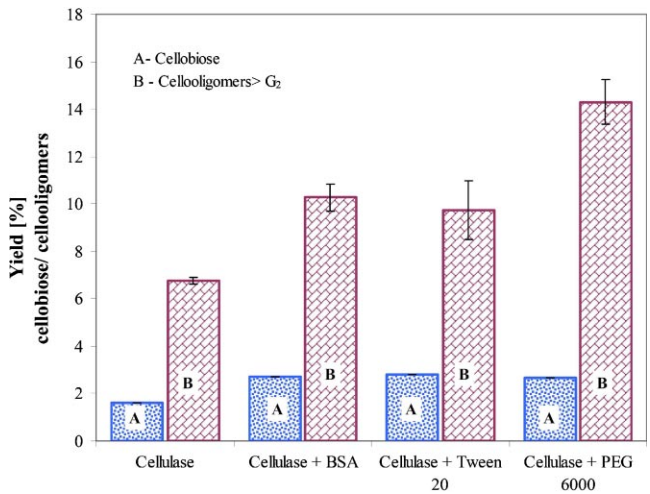

Figure 10. The effect of 600 mg additives/g glucan on cellobiose (A) and higher cellooligomer (B) yields following 72 h of enzymatic hydrolysis of Avicel glucan with a combined cellulase plus β-glucosidase mass loading of 16.1 mg/g glucan. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

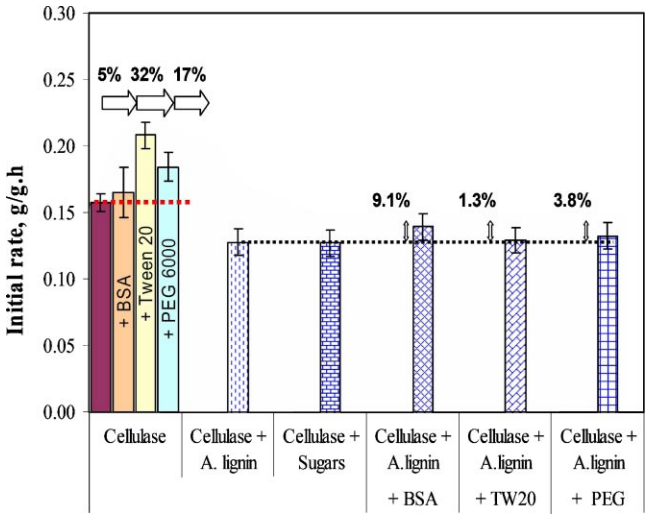

Figure 11. Effect of 300 mg of BSA, Tween-20, and PEG6000 per g glucan on the initial rate of Avicel hydrolysis to glucose in the presence of about 0.9 g/L of soluble lignin, 2.0 g/L glucose, or 10 g/L xylose and with a combined cellulase plus β-glucosidase mass loading of 32.2 mg/g glucan. The controls on the left are the results for enzymatic hydrolysis without adding lignin or sugars and list the gains in yields for each additive. A.lignin* = acid soluble lignin. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

technologies, but the magnitude of the effect varied with the type of pretreatment, as summarized in Table II and Figure 12a and b. Table II clearly shows that the increase in glucose and xylose yield with additives was much higher than with double the amount of enzymes for the control without additives (16.1 mg/g glucan) for all pretreatments except dilute acid with Sunds system and controlled pH. Figure 12 shows that, among leading pretreatments, ARP, SO_2, lime, and dilute acid with the Parr reactor gained most from additives, however additives had the least benefit for untreated corn stover and corn stover pretreated with dilute acid with the Sunds system and controlled pH. In addition, although xylose yields were low for all pretreatments even with additives, additives had the least impact on xylose release from AFEX pretreated solids, and it appears that supplementation of cellulase with xylanase and additives should realize high total sugar yields. For ARP pretreated solids, all additives apparently showed similar enhancements in sugar yields of about 55–65%.

Conclusions

The effect of additives and their loadings on glucose and xylose release from solids prepared by leading pretreatment options that span a range of pH values was evaluated at a moderate mass loading of cellulase. Of the additives evaluated, BSA enhanced glucose release the most for untreated corn stover (20%) and AFEX (14%) and dilute acid (5%) pretreated solids, although the maximum increase in xylose release using BSA was with untreated corn stover (36.9%) and dilute acid (11.4%) and SO_2 (59%) pretreated solids. However, Tween-20 was particularly effective with Avicel glucan (63.5% enhancement at a loading of 600 mg Tween-20/g glucan), glucan for both lime (19%) and SO_2 (63%) pretreated solids, and xylan in dilute acid (11.4%), lime (73.2%), and SO_2 (73.2%) pretreated solids. But PEG6000 improved sugar release less than the other two additives for all pretreatments except ARP. Furthermore, for all pretreatments except lime and SO_2 with Tween-20, additive loadings above 150 mg additives/g glucan had little additional effect on sugar release. Consistent with this finding, Eriksson and coworkers reported (Borjesson et al., 2007; Eriksson et al., 2002) that the loading of additives beyond a certain level had limited impact on sugar release. However, although no synergy was observed between/among additives for all pretreatments except SO_2, it may be more beneficial to use the mixture of these additives to realize a high total sugar yield.

The highest percentage increase in total sugar yields was for SO_2 pretreated solids with Tween-20 (122%) followed by

Table II. Maximum percentage increase in sugars yields possible and maximum percentage increase obtained with additives and twice enzyme loading of control (16.1 mg/g glucan) for enzymatic hydrolysis of Avicel glucan, untreated corn stover, and corn stover pretreated by leading pretreatment technologies.

	Maximum possible percentage increase in yield			% Increase in yield obtained with a cellulase mass loading twice the control (32.2 mg/ g glucan)			% Increase in yield obtained with additives [additive-loading]		
Pretreatment	Glucose	Xylose	Total sugar	Glucose	Xylose	Total sugar	Glucose	Xylose	Total sugar
Avicel	72.9	—	72.9	25.7	—	25.7	63.5 [T-C]	—	63.5 [T-C]
Untreated CS	431.9	1443.2	1875.1	2.1	6.5	8.6	20.0 [BS-A]	36.9 [BS-A]	56.9 [BS-A]
SO2	72.4	129.3	201.7	38.8	33.0	71.8	63.0 [T-C]	59.0 [T-C]	122 [T-C]
Dilute acid (Sunds system)	108.1	31.7	139.8	17.5	6.8	24.3	5.0 [BS-A]	11.4 [T-C]	16.1 [T-C]
Dilute acid (Parr reactor)	36.7	18.4	55.1	32.8	9.0	41.7	36.7 [P-C]	18.4 [P-C]	55.1 [P-C]
Controlled pH	42.8	130.9	173.7	19.8	34.9	54.7	12.8 [BS-A]	21.0 [T-C]	28.8 [T-C]
AFEX	29.3	49.7	79	13.1	12.0	25.1	14.0 [BS-C]	10.0 [T-C]	22.8 [T-C]
ARP	54.9	71.4	126.3	39.5	20.9	60.3	64.0 [P-C]	58.4 [BS-A]	114.2 [P-C]
Lime	40.0	172.9	212.9	17.4	29.6	47.1	19.0 [T-C]	73.2 [T-C]	92.2 [T-C]

BS, bovine serum albumin (BSA), T, Tween-20, P, PEG6000; A, 150 mg/g glucan, B, 300, mg/g glucan, C, 600 mg/g glucan.

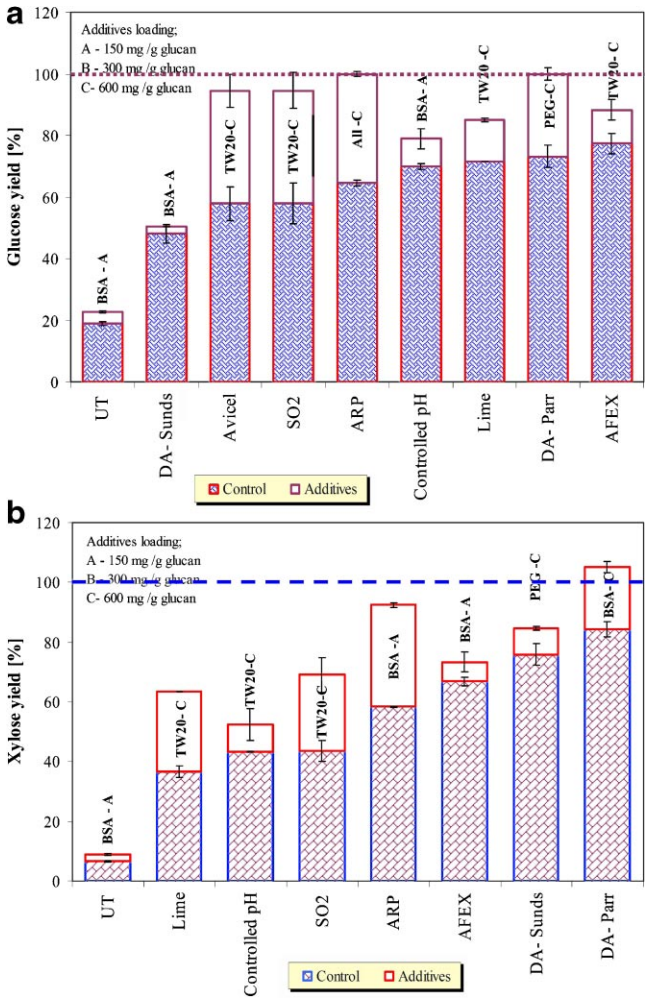

Figure 12. Summary of additives and corresponding loading that had the greatest impact on (**a**) glucose and (**b**) xylose release for each pretreatment technology following 72 h of enzymatic hydrolysis at a combined cellulase plus β-glucosidase mass loading of 16.1 mg/g glucan. *The enzyme loading for dilute acid pretreated solids with Parr reactor was 6.2 mg/g glucan. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

ARP pretreated solids with PEG6000 (114%) and lime pretreated solids with Tween-20 (92%). Thus, enhancement in sugar release by additives depends on the type of pretreatment. The effectiveness of the additives was observed to depend on the type of sugars left in the solids for some pretreatments, as shown in Table II.

In addition to reduction of unproductive binding of enzymes to lignin, as reported in the literature, additives may significantly impact enzyme activity and/or availability by impeding unproductive binding with carbohydrates and increasing stability as indicated by the presence of higher amounts of soluble cellobiose and higher cellooligomers after 72 h of hydrolysis for Avicel glucan. Additives also appeared to be more effective for longer hydrolysis times. The inhibition of enzymatic hydrolysis by acid soluble lignin appeared to result more from the sugars in the lignin solution than from lignin, with additives providing some relief. Taken together, these results suggest that at least a portion of the benefits of additives could be through reducing end product inhibition of enzymes.

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