



Investigation of enzyme formulation on pretreated switchgrass

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ABSTRACT

This work studied the benefits of adding different enzyme cocktails (cellulase, xylanase, β -glucosidase) to pretreated switchgrass. Pretreatment methods included ammonia fiber expansion (AFEX), dilute-acid (DA), liquid hot water (LHW), lime, lime + ball-milling, soaking in aqueous ammonia (SAA), and sulfur dioxide (SO₂). The compositions of the pretreated materials were analyzed and showed a strong correlation between initial xylan composition and the benefits of xylanase addition. Adding xylanase dramatically improved xylan yields for SAA (+8.4%) and AFEX (+6.3%), and showed negligible improvement (0–2%) for the pretreatments with low xylan content (dilute-acid, SO₂). Xylanase addition also improved overall yields with lime + ball-milling and SO₂ achieving the highest overall yields from pretreated biomass (98.3% and 93.2%, respectively). Lime + ball-milling obtained an enzymatic yield of 92.3 kg of sugar digested/kg of protein loaded.

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1. Introduction

Because of its high abundance and relatively low cost, lignocellulosic biomass is a promising source of renewable liquid fuels (Klyosov, 1986; Saha and Cotta, 2008). Sources of lignocellulosic biomass include energy crops, agricultural crop residues, industrial waste, and municipal paper waste (Zaldivar et al., 2001). It is composed mainly of cellulose and hemicellulose, which when hydrolyzed provide a source of carbohydrates for ethanol fermentation. However, the hydrolysis of cellulose and hemicellulose is one of the main hurdles to fully realizing the potential of cellulosic ethanol. Some of the key chemical and physical barriers which limit enzymatic hydrolysis include: high lignin content, cellulose crystallinity, degree of cellulose polymerization, low surface area, and presence of acetyl groups on hemicellulose (McMillan, 1994; Sun and Cheng, 2002). The goal of pretreatments, both chemical and

physical, is to remove some of these barriers and render the biomass more susceptible to enzymatic digestion.

This study was a collaborative effort between members of the Consortium for Applied Fundamentals and Innovation (CAFI), which was formed to compare different pretreatment technologies using consistent materials and analytical methods (Mosier et al., 2005; Wyman et al., 2005a). The goals of CAFI I and II were to determine optimal conditions for varying pretreatment technologies for corn stover (Wyman et al., 2005b) and poplar wood (Wyman et al., 2009), respectively. This study was part of CAFI III, which focuses on increasing enzymatic digestibility of switchgrass, a promising bioenergy crop with high biomass yield, moisture efficiency, low nutrient requirement, and stand longevity (Samson and Omielan, 1994). It can grow in many environments, including most regions of the United States (Gould, 1968) and is a promising substrate for ethanol production (Schmer et al., 2006; Wright and Turhollow, 2010). The primary contributors to this study were Auburn University (soaking in aqueous ammonia pretreatment), Michigan State University (ammonia fiber expansion pretreatment), Purdue University (liquid hot water

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pretreatment), Texas A&M University (lime pretreatment and data analysis), and University of California Riverside (sulfur dioxide and dilute-acid pretreatments).

To determine pretreatment effectiveness and optimum pretreatment conditions, the primary analytical tool utilized by the CAFI team is enzymatic hydrolysis. A significant amount of work has been devoted to studying the effects of cellulase and β -glucosidase on pretreated substrates (Alvira et al., 2010; Cardona et al., 2010; Wyman et al., 2009). With the high cost of feedstock, pretreatment, and enzymes, it is necessary to optimize the enzymatic hydrolysis of both cellulose and hemicellulose (Chandra et al., 2008; Girio et al., 2010; Gnansounou and Dauriat, 2010; O'Dwyer et al., 2007). The primary goal of this project was to explore the effect of adding a third enzyme, xylanase, to the standard enzyme mixture of cellulase and β -glucosidase. Xylanase is primarily responsible for hydrolyzing hemicellulose by cleaving β -1,4 xylan bonds. Changes in enzymatic digestibility due to xylanase addition were observed by measuring both individual and overall carbohydrate yields. While holding β -glucosidase constant, varying the ratio of cellulase to xylanase achieved an optimal ratio that maximized overall yields while reducing total enzyme loading. A secondary goal of the project was to study the effect of overall yield in the absence of β -glucosidase, which would determine the need to add β -glucosidase when both cellulase and xylanase were present.

2. Methods

2.1. Substrate and enzymes

The feedstock used in this study was the Dacotah variety of switchgrass (*Panicum virgatum*) kindly provided by Ceres, Inc. This variety was planted on December 6, 1999 in Pierre, SD and harvested on March 1, 2008 after the plot stood over the winter. The bales were stored indoors until shipped to Hazen Research, Inc. (Golden, CO) where they were ground by a hammer mill equipped with a $\frac{1}{4}$ -in screen. The material was then mixed using the cone and quartering technique, separated into 5-kg sub-lots and divided amongst the CAFI members. The composition determined by Ceres, Inc. was 35.0% glucan, 21.8% xylan, 3.5% arabanin, 21.4% lignin, 2.8% acetyl, and 8.1% extractives. Each CAFI laboratory further reduced the particle size to pass through 40 (ASTM) mesh. After reducing the particle size, the switchgrass was washed with hot water. Dry switchgrass (200 g) was mixed with 2 L of 80–90 °C distilled water and allowed to stand 10–15 min. The slurry was vacuum filtered using Whatman No. 41 filter paper. The mixing and filtration was performed three times followed by drying the washed solids in a 45 °C oven. The composition of the washed material as measured by Texas A&M University was 37.2% glucan, 23.8% xylan, 2.5% arabanin, and 20.8% lignin.

Cellulase was Spezyme CP[®] (lot 301–04075-054, 82 mg protein/mL, 59 FPU/mL). Hemicellulase was Multifect xylanase[®] (lot 301–04021-015, 27 mg protein/mL). Both cellulase and hemicellulase were kindly provided by Genencor International, Inc[®]. The β -glucosidase was Novozyme 188[®] (67 mg protein/mL, 600 CBU/mL) and was obtained from Sigma–Aldrich (St. Louis, MO). The protein concentration of each enzyme was measured using TCA precipitation and was reported by Genencor (Spezyme CP and Multifect Xylanase) and Michigan State University (Novozyme 188).

2.2. Pretreatment methods

2.2.1. Ammonia fiber expansion (AFEX)

The AFEX pretreatment conditions were chosen to limit hemicellulose degradation. The pretreatment was performed in a 1.5-L

stainless steel (#316) Parr reactor. Distilled water was added to the switchgrass at a loading ratio of 2 g H₂O/g dry biomass and the slurry was added to the preheated (150 °C) reactor. The reactor was sealed and evacuated using a rotary vacuum pump while ammonia was heated in a separate pressurized vessel. Once heated, the ammonia was added to the reactor at a loading of 1.5 g NH₃/g dry biomass. The pretreatment ran for 30 min with a maximum temperature of 155–165 °C, which decreased to a final temperature between 104 and 119 °C. The reactor was then rapidly vented and the biomass was removed. The biomass was stored overnight in a fume hood to allow evaporation of residual ammonia.

2.2.2. Dilute sulfuric acid (DA)

Switchgrass (50 g) was presoaked in 10-g/L dilute sulfuric acid overnight at room temperature with a solid loading of 10 wt.%. The pretreatment was performed in a 1-L Parr reactor made of Hastelloy C. Heating was provided by a 4-kW fluidized sand bath with stirring (200 rpm) using two 40-mm-diameter stacked pitched-blade impellers. Pretreatment was run at 140 °C for 40 min, which did not include an additional 2-min heating time. The reactor was quenched in a room-temperature water bath until the temperature dropped to 80 °C. The pretreatment slurry was vacuum filtered through a glass fiber filter with the temperature consistently greater than 60 °C. The resulting solids were washed with room-temperature deionized water until the filtrate pH was greater than 6.0.

2.2.3. Lime

Lime pretreatment was conducted in a pair of 304 stainless steel pipe reactors (5-in long, 1.5-in ID) with 1.5-in 304 stainless steel caps. The reactors were sealed using Teflon tape. Reactors were loaded with 8 g dry switchgrass each and excess calcium hydroxide (1 g Ca(OH)₂/g dry biomass) and water (15 g/g dry biomass). Constant 6.89-bar pure oxygen was supplied to a manifold through a flexible stainless steel hose attached to an oxygen tank. The reactors were connected to a swing arm to provide constant stirring and placed in a preheated temperature-controlled oven at 120 °C. The reaction time was 4 h after which the reaction was quenched by removing the reactors from the oven and immediately placing them in an ice bath. Once cooled, the reactors were opened slowly to relieve pressure, and the contents were transferred to a 1-L plastic centrifuge bottle using distilled water. The slurry was neutralized using 5-N HCl to a pH of approximately 4.0, and then underwent several washings with distilled water until the pH of the slurry rose to approximately 6.0. The final slurry was vacuum filtered and the filtrate was collected for carbohydrate analysis. Moisture content and final solid weight were recorded to obtain pretreatment yield and the solids were stored in the freezer until compositional analysis and enzymatic hydrolysis were performed.

2.2.4. Lime + ball-milling

Lime pretreatment followed the same procedure as above. The pretreated solids were thoroughly dried (moisture content <10%) before ball-milling in a 300-mL porcelain jar loaded with 0.375-in zirconia grinding medium. The grinding medium was loaded to fill 50% of the jar volume (approximately 258 g) and biomass was loaded at a ratio of 43 g grinding medium/g dry biomass. The jars were sealed and placed on rollers rotating at 68 rpm for 3 days.

2.2.5. Liquid hot water (LHW)

Switchgrass was mixed with deionized water at a loading ratio of 15 wt.%. The pretreatment reactor was stainless steel (#316) tubing (1-in OD \times 0.083-in wall thickness, 4.5-in length, 45-mL to-

tal volume) capped at each end with a 1-in tube end fitting. Sample volume was chosen to be 33.7 mL to allow 25% headspace for liquid expansion. The reaction was run at 200 °C for both 5 and 10 min. The reactor was heated in a Tecam SBL-1 fluidized sand bath with a heat-up time of 8 min, which was not included in reaction time. Upon completion, the pretreatment was quenched by placing the reactor in water for 10 min.

2.2.6. Soaking in aqueous ammonia (SAA)

The SAA pretreatment was performed in a stainless steel batch reactor (1.375-in ID × 6-in long). Switchgrass (10 g) was loaded with 90 mL 15% NH₄OH. The reactor was placed in a preheated temperature-controlled oven at 160 °C for 60 min. Heat-up time was 20 min and was not included in the reaction time. The reactor was quenched in a room-temperature water bath. The pretreatment slurry was vacuum filtered and the solids were washed using deionized water until the pH was approximately 6.0.

2.2.7. Sulfur-dioxide (SO₂)

Moist (approximately 65% moisture) switchgrass was impregnated overnight with 5 wt.% gaseous SO₂ (>99% pure) at room temperature in a sealed heavy-duty Ziploc bag. The impregnated switchgrass was transferred to a 1-L Hasteloy C Parr reactor and mixed with deionized water to a solid loading of 10 wt.% on a dry basis. The reaction was run at 180 °C for 10 min in a 4-kW fluidized sand bath. Stirring was provided by two 40-mm-diameter stacked pitched blade impellers at 200 rpm. Heat-up time was 2 min and was not included in reaction time. The reactor was quenched in a water bath until the reactor temperature dropped to 80 °C. The pretreatment slurry was immediately vacuum filtered while maintaining a temperature greater than 60 °C. The resulting solids were washed with deionized water until filtrate pH was greater than 6.0.

2.3. Compositional analysis

Compositional analysis was performed on the raw, pre-washed, and pretreated samples. The material was prepared by air drying to a measured moisture content of less than 10%. The composition was analyzed using an NREL acid hydrolysis procedure (Sluiter et al., 2008). The sample (0.3 g) was weighed into a glass test tube followed by adding 3 mL of 72 wt.% sulfuric acid. The test tubes were placed in a 30 °C water bath and stirred regularly for 1 h. The contents of the test tube were quantitatively transferred to glass autoclave bottles using 84 mL distilled water, capped, sealed, and steam autoclaved for 1 h. Samples were cooled, opened, and filtered through glass filtering crucibles, which were placed in a 105 °C oven to dry. The filtrate was neutralized and then analyzed for carbohydrates using HPLC Analysis (Bio-Rad Aminex HPX-87P column, HPLC grade water mobile phase, 0.6 mL/min, 80–85 °C column temperature). The weight of the dried, filtered solids minus their ash weight was recorded and used to calculate lignin content. Ash content was determined by heating samples in a 575 °C furnace until completion. The extractives were determined by extracting the biomass with 95% ethanol for 24 h in a Soxhlet apparatus. The measured compositions for both the raw and pretreated materials were used in the enzymatic hydrolysis loading calculations.

2.4. Enzymatic hydrolysis

The enzymatic hydrolysis procedure for both glucan and xylan closely followed the enzymatic saccharification procedure provided by NREL (Selig et al., 2008). Hydrolysis samples were prepared in 50-mL plastic centrifuge tubes. Pretreated biomass loading weight was calculated based on moisture content and glu-

can composition to yield 0.1 g glucan per sample. Sodium citrate buffer (5 mL, 0.1-M, pH 4.8), 0.04 mL tetracycline (10 mg/mL in 70% ethanol), 0.03 mL cycloheximine (10 mg/mL in distilled water), 1 mL of each enzyme dilution (cellulase, xylanase, β-glucosidase), and an appropriate volume of water were added to bring the total working volume to 10 mL. The enzyme dilutions were calculated on a raw glucan basis using the enzyme activity and desired enzyme loading. Hydrolysis occurred in a shaking incubator (100 rpm) at 50 °C for 72 h. To quench the hydrolysis, the samples were either placed in a 105 °C oven or in boiling water for 5–10 min and then cooled in an ice bath. Samples were stored in a freezer until HPLC analysis. HPLC analysis (Bio-Rad Aminex HPX-87P column, HPLC grade water mobile phase, 0.6 mL/min, 80–85 °C column temperature) was used to measure the glucose and xylose concentrations of each sample. These concentrations were then recalculated as glucan and xylan concentrations to report digestibility yields.

2.5. Experimental design

Substrate preparation, pretreatments, compositional analysis, and enzymatic hydrolysis were all performed by each individual CAFI laboratory. The compositional analysis and enzymatic hydrolysis results of each pretreatment type were then sent to Texas A&M University. Texas A&M University analyzed carbohydrate yields to determine the most effective enzyme ratio for each pretreatment. For each pretreatment, the experiment measured the enzymatic digestibility of 23 different samples (Table 1). The 23 samples were comprised of enzyme loadings in two sets: Set A (13.4, 33.4, 78.4, 123.4, and 243.4 mg protein/g raw glucan) and Set B (30.0 mg protein/g raw glucan). In Set A, β-glucosidase was held constant (3.4 mg protein/g raw glucan), to be consistent with previous CAFI research (Wyman et al., 2005b). These enzyme concentrations were chosen to represent enzyme loadings ranging from very low (economical) to very high (gross excess). Furthermore, little information was available on the effects of cellulase:xylanase loading ratio, so for each of these five total enzyme loadings, four cellulase:xylanase ratios were employed (1:0, 5:1, 2:1, and 1:1). Set B employed three cellulase:xylanase ratios (5:1, 2:1, 1:1), but no β-glucosidase.

Table 1
Enzyme loadings.

Sample	Cellulase (mg/g raw glucan)	Xylanase (mg/g raw glucan)	B-glucosidase (mg/g raw glucan)	Total Enzyme (mg/g raw glucan)
1A	10.0	0.0	3.4	13.4
2A	8.3	1.7	3.4	13.4
3A	6.7	3.3	3.4	13.4
4A	5.0	5.0	3.4	13.4
5A	30.0	0.0	3.4	33.4
6A	25.0	5.0	3.4	33.4
7A	20.0	10.0	3.4	33.4
8A	15.0	15.0	3.4	33.4
9A	75.0	0.0	3.4	78.4
10A	62.5	12.5	3.4	78.4
11A	50.0	25.0	3.4	78.4
12A	37.5	37.5	3.4	78.4
13A	120.0	0.0	3.4	123.4
14A	100.0	20.0	3.4	123.4
15A	80.0	40.0	3.4	123.4
16A	60.0	60.0	3.4	123.4
17A	240.0	0.0	3.4	243.4
18A	200.0	40.0	3.4	243.4
19A	160.0	80.0	3.4	243.4
20A	120.0	120.0	3.4	243.4
21B	25.0	5.0	0.0	30.0
22B	20.0	10.0	0.0	30.0
23B	15.0	15.0	0.0	30.0

3. Results and discussion

3.1. Composition of pretreated samples

Table 2 shows the compositional analysis of the eight pretreated materials plus raw and washed feedstocks. The washing procedure before pretreatment did not greatly affect the composition with just a slight increase (1.5%) in glucan composition. Lime and SAA pretreatments both reduced lignin content of the washed feedstock, by 7.2% and 12.4%, respectively. There was significant removal of xylan; while, the glucan content of the lime pretreatment significantly increased because of the weight loss after pretreatment. The dilute-acid and SO₂ pretreatments both had high levels of xylan reduction, which resulted in a significant increase in glucan content and a slight increase in lignin. The compositions of the AFEX and LHW pretreatments were relatively unchanged from the raw switchgrass composition. (Note: AFEX, LHW 5 min, and LHW 10 min samples were not washed after pretreatment.)

3.2. Effect of xylanase addition on carbohydrate yields

The primary goal of this study was to determine the optimum ratio of cellulase to xylanase that maximizes overall carbohydrate yield. For each pretreatment, 20 samples were analyzed which consisted of five total enzyme loadings (13.4, 33.4, 78.4, 123.4, and 243.4 mg protein/g raw glucan) with four different cellulase:xylanase ratios per enzyme loading (1:0, 5:1, 2:1, 1:1). After hydrolysis, the glucan, xylan, and overall yield were calculated based on pretreated compositions using the following definitions:

$$\text{Glucan yield} \equiv \frac{\text{glucan digested}}{\text{initial glucan loaded}}$$

$$\text{Xylan yield} \equiv \frac{\text{xylan digested}}{\text{initial xylan loaded}}$$

$$\text{Overall yield} \equiv \frac{\text{glucan digested} + \text{xylan digested}}{\text{initial glucan loaded} + \text{initial xylan loaded}}$$

Fig. 1 shows the glucan yield, xylan yield, and overall yield results. (Note: Only the best performing enzyme loading ratios are shown.) Each pretreatment has a different carbohydrate composition, so the effect of cellulase:xylanase ratio showed a different result for each pretreatment method. In most cases, there was not a significant increase in overall yield once the total enzyme loading was greater than 78.4 mg protein/g raw glucan. All future discus-

Table 2

Composition and pretreatment yields. Note: AFEX and LHW were not washed after pretreatment.

	Glucan(%)	Xylan(%)	Lignin(%)	Other(%)	Pretreatment Yield(g treated biomass/100 g raw biomass)
Raw	35.0	21.8	21.4	21.8	–
Washed	36.5	22.7	20.7	20.1	92.6
Lime + ball-mill	48.6	18.7	13.5	19.2	69.2
Lime	48.6	18.7	13.5	19.2	69.2
AFEX	35.9	22.5	24.4	17.2	95.1
SAA	34.5	13.6	8.3	43.6	62
LHW5 min	36.5	22.7	20.7	20.1	92.6
LHW10 min	36.5	22.7	20.7	20.1	92.6
DA	50.6	7.3	28.6	13.5	60.9
SO ₂	58.7	4.5	27.6	9.2	57.3

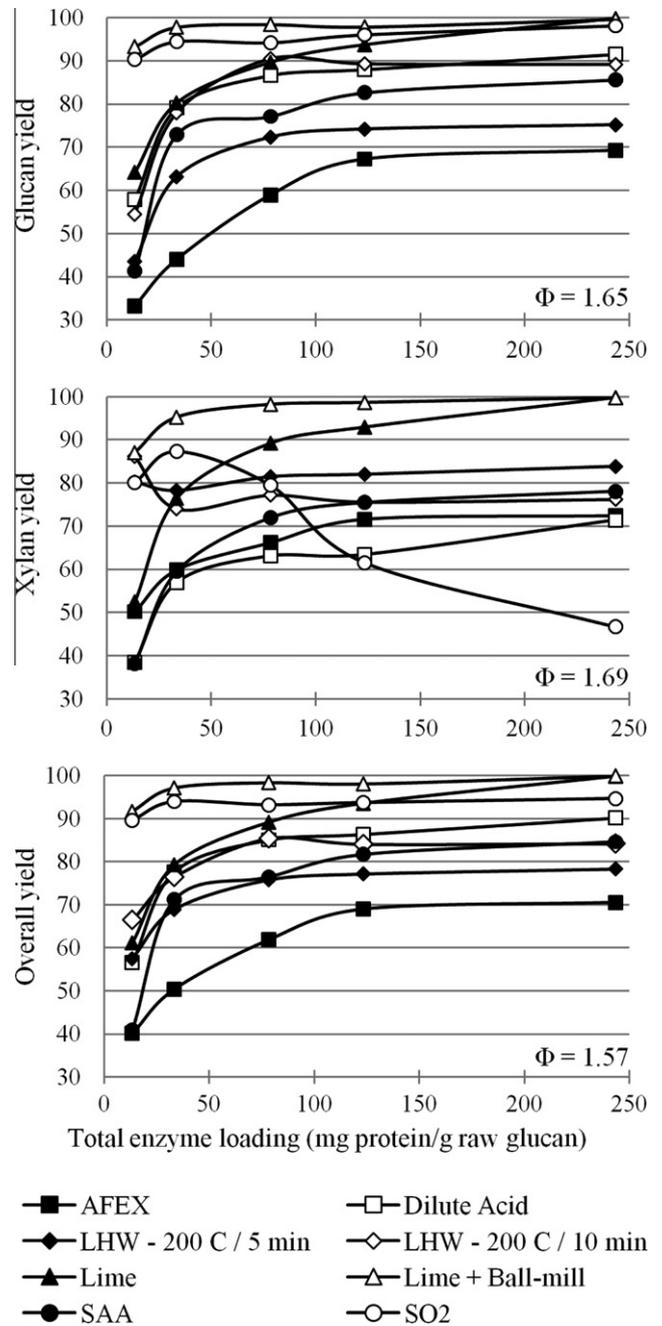


Fig. 1. Glucan, xylan, and overall enzymatic yield (g component digested/100 g treated component) as a function of total enzyme loading (mg protein/g raw glucan). Best performing enzyme loading ratios are shown. Enzymatic hydrolysis conditions: 72 h, 50 °C, and enzymes were loaded on a raw glucan basis. Φ = Average standard deviation.

sions will focus on an enzyme loading of 78.4 mg protein/g raw glucan. All values are given in relation to a percent increase or decrease over pure Spezyme CP, the control.

For AFEX pretreatment, xylanase addition noticeably improved xylan yield (+6.3%) and glucan yield (+4.6%). Further increasing the xylanase ratio improved xylan yield with increases of 8.1% (2:1) and 9.1% (1:1). At 78.4 mg protein/g raw glucan, the overall yield was 61.9% (1:1). [Note: AFEX was the only pretreatment that benefitted from higher enzyme loadings. At 243.4 mg protein/g raw glucan it reached a maximum overall yield of 72.1% at the 1:1 ratio.]

For SAA pretreatment, xylanase addition dramatically increased xylan yield (8.4%) With increased xylanase, xylan digestibility improved 13.1% (2:1) and 17.9% (1:1). At a total protein loading of 78.4 mg/g raw glucan, the maximum glucan yield was 77.1% whereas the maximum xylan yield was 72.0% (1:1). The SAA pretreatment contained only 13.6% xylan in its initial composition. Although xylanase addition significantly increased xylan yield, the increase in overall yield was more moderate, 2.6% (5:1), 3.9% (2:1), and 5.2% (1:1). A maximum overall yield of 76.5% (1:1) was achieved.

Compared to AFEX and SSA pretreatments, lime pretreatment had slightly less benefit from xylanase addition with a 4.9% xylan yield increase (5:1). Increased xylanase addition improved xylan yields by 5.4% (2:1) and 7.1% (1:1). Some increase in glucan yield (2.7%, 1.8%, 2.5%) and overall yield (3.3%, 2.7%, 3.7%) was also observed for the 5:1, 2:1, and 1:1 ratios, respectively. Lime pretreatment obtained a maximum overall yield of 89.6% (1:1).

Ball-milling the lime pretreated sample diminished the benefits of xylanase addition with improved xylan yields of 2.1% (5:1), 2.6% (2:1), and 1.5% (1:1). It outperformed all other pretreatments with a maximum overall yield of 98.3% (2:1).

The 200 °C/5-min LHW pretreatment slightly benefitted from xylanase addition with an increased xylan yield of 1.8% (5:1), 2.2% (2:1), and 1.5% (1:1). The most promising ratio (2:1) improved glucan yield 2.4% and overall yield 2.3%. At this ratio, the maximum overall yield was 75.8%. For the 200 °C/10-min LHW pretreatment, only the 5:1 ratio increased xylan yield (2.6%). The 2:1 ratio showed negligible improvement and the 1:1 decreased xylan yield (−0.9%). The maximum overall yield was 85.4% (5:1).

The SO₂ pretreatment caused xylan yield changes of −0.4% (5:1), −1.2% (2:1), and 2.9% (1:1). The best-performing ratio (2:1) had an overall yield of 93.2%.

For dilute-acid pretreated switchgrass, the effect of xylanase addition was negligible with changes in xylan yields of −1.0% (5:1), 0% (2:1), and 0.9% (1:1). Glucan yield and overall yield decreased with increased xylanase addition. The maximum overall yield (91.2%) was achieved using just Spezyme CP.

Standard deviations were minimal for glucan, xylan, and overall yields of the AFEX, SSA, lime, and lime + ball-milling pretreatments. The yields of the LHW pretreatments produced higher standard deviations, making it difficult to determine if there was an improvement with added xylanase. Unfortunately, standard

deviation was not provided for the SO₂ and dilute-acid pretreatments.

The optimum enzyme loading ratio was 1:1 cellulase:xylanase for the AFEX, SAA, and lime pretreatments. Lime + ball-mill, 200 °C/5-min LHW, and SO₂ pretreatments obtained maximum yields at an optimum ratio of 2:1. The optimum ratios for the 200 °C/10-min LHW and dilute-acid pretreatments were 5:1 and 1:0, respectively. The difference in optimal enzyme loading ratios is highly dependent on the pretreated composition. AFEX and lime pretreatments, for example, had higher initial xylan compositions than dilute-acid and SO₂ and thus favored higher xylanase loadings.

3.3. Enzymatic yield

The enzymatic yield is defined as the ratio of total carbohydrates digested per unit of protein loaded.

$$\text{Enzymatic yield} \equiv \frac{\text{total glucan + xylan digested}}{\text{total protein loaded}}$$

Enzymatic yield is a useful tool to determine the optimal enzyme loading which results in high sugar yields while minimizing the use of costly enzymes. Fig. 2 shows enzymatic yield as a function of total protein loading. As total protein loading increases, there are diminishing returns in overall yield.

Enzymatic yield can be used to compare the effectiveness of each pretreatment. At the lowest enzyme loading (13.4 mg protein/g raw glucan), Fig. 2 shows that lime pretreatment has a maximum enzymatic yield of 64.2 g of sugar digested/g protein loaded. When ball-milling is added to the lime pretreatment, the enzymatic yield at the same enzyme loading, significantly increased to 91.3 g of sugar digested/g protein loaded. With knowledge of the cost of enzymes and of the mechanical process, the economic viability of using the mechanical process could be determined.

Fig. 3 compares enzymatic yield to overall yield. When designing a hydrolysis system, a typical goal is to achieve a target overall yield. From this plot, a desired overall yield specifies the enzymatic yield, which determines the required amount of enzyme for a desired mass of sugar.

Fig. 3 shows the overall yield vs. enzymatic yield plots for all four enzyme ratios (Spezyme CP, 5:1, 2:1, 1:1). The benefit of xylanase addition is readily apparent when comparing the

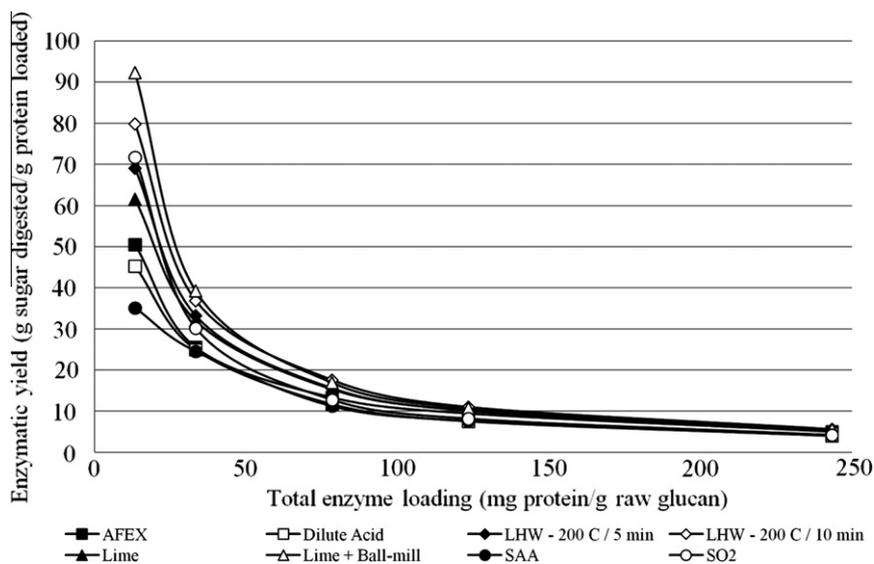


Fig. 2. Enzymatic yield as a function of total protein loading.

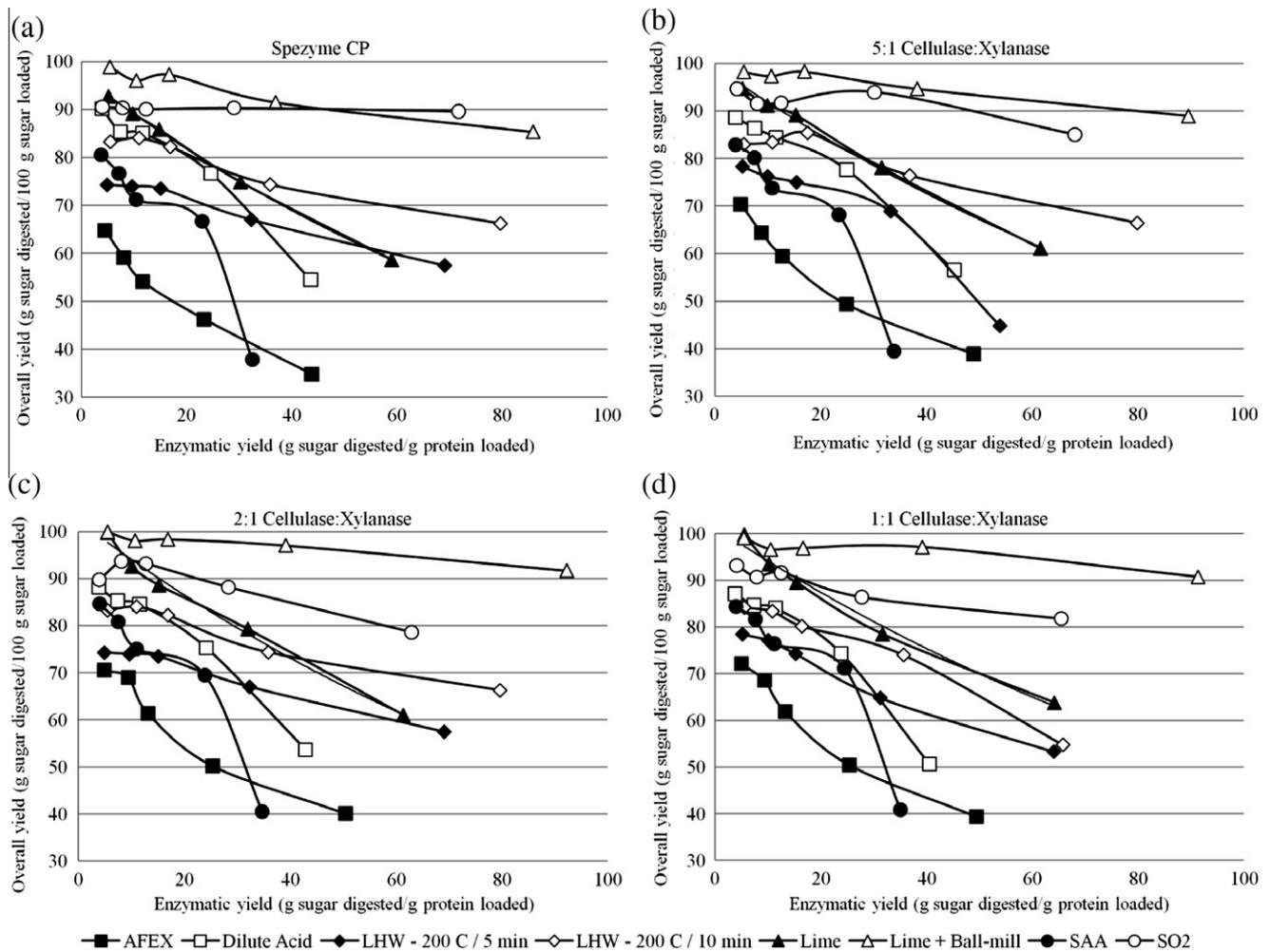


Fig. 3. Enzymatic yield vs. overall yield.

Spezyme CP plot (Fig. 3a) to the 5:1, 2:1, and 1:1 plots (Fig. 3b–d). There is a very noticeable upward shift in all plots when xylanase is added. Using lime pretreatment as an example, at a target overall yield of 80%, an enzymatic yield value of 22.6 g sugar digested/g protein is obtained for the Spezyme CP case. When xylanase is added to the enzyme cocktail, at the same target overall yield, the enzymatic yield increases to 27.6 g sugar digested/g protein. In these plots, it is also clear that lime + ball-milled pretreatment and SO₂ pretreatment were so effective at increasing digestibility that regardless of enzymatic yield, they obtain high overall yields.

3.4. Economic study

According to a recent technoeconomic analysis on an enzymatic ethanol process (Aden and Foust, 2009), the current (2009) minimum ethanol selling price is \$2.34/gal with the goal to obtain a minimum ethanol selling price of \$1.33/gal ethanol by 2012. To achieve this, the author states that enzyme cost must be significantly reduced, while increasing enzymatic activity and pretreatment effectiveness. The enzymatic and overall yields measured in this study were used to estimate the current state of technology. (Note: One limit to this model is the assumption that the 1% glucan loading used for enzymatic hydrolysis is comparable to a commercially relevant glucan loading.) The current estimated cost of raw biomass is approximately \$60/ton (\$0.06/kg) with the price decreasing to \$46/ton (\$0.05/kg) by 2012 (Aden and Foust, 2009). Enzyme cost estimates are not readily available so Fig. 4 shows

cost contributions for a range of enzyme costs. Feedstock cost per liter of ethanol can be calculated as a function of raw biomass cost, pretreatment yield, pretreatment composition, overall yield, theoretical fermentation yield, actual yield, and ethanol density. Enzyme cost per liter of ethanol can be estimated using the cost of enzymes, enzymatic yield, theoretical fermentation yield, actual yield, and ethanol density. Assuming \$0.06/kg biomass, \$4.41/kg enzyme, 69.2% pretreatment yield, and 90% fermentation yield, a sample calculation using lime + ball-milling follows:

Total sugar composition

$$0.486 \text{ kg glucan} \left(\frac{\text{kg glucose}}{0.9 \text{ kg glucan}} \right) + 0.187 \text{ kg xylan} \left(\frac{\text{kg xylose}}{0.88 \text{ kg xylan}} \right)$$

kg pretreated biomass

$$= \frac{0.753 \text{ kg sugar}}{\text{kg pretreated biomass}}$$

Feedstock cost

$$\begin{aligned} & \frac{\$0.06}{\text{kg raw biomass}} \times \frac{\text{kg raw biomass}}{0.692 \text{ kg pretreated biomass}} \\ & \times \frac{\text{kg pretreated biomass}}{0.753 \text{ kg sugar}} \times \frac{\text{kg sugar}}{0.917 \text{ kg digested sugar}} \\ & \times \frac{\text{kg digested sugar}}{0.51 \text{ kg EtOH}} \times \frac{1}{0.9} \times \frac{0.791 \text{ kg EtOH}}{\text{L EtOH}} \\ & = \frac{\$0.24}{\text{L EtOH}} = \frac{\$0.90}{\text{gal EtOH}} \end{aligned}$$

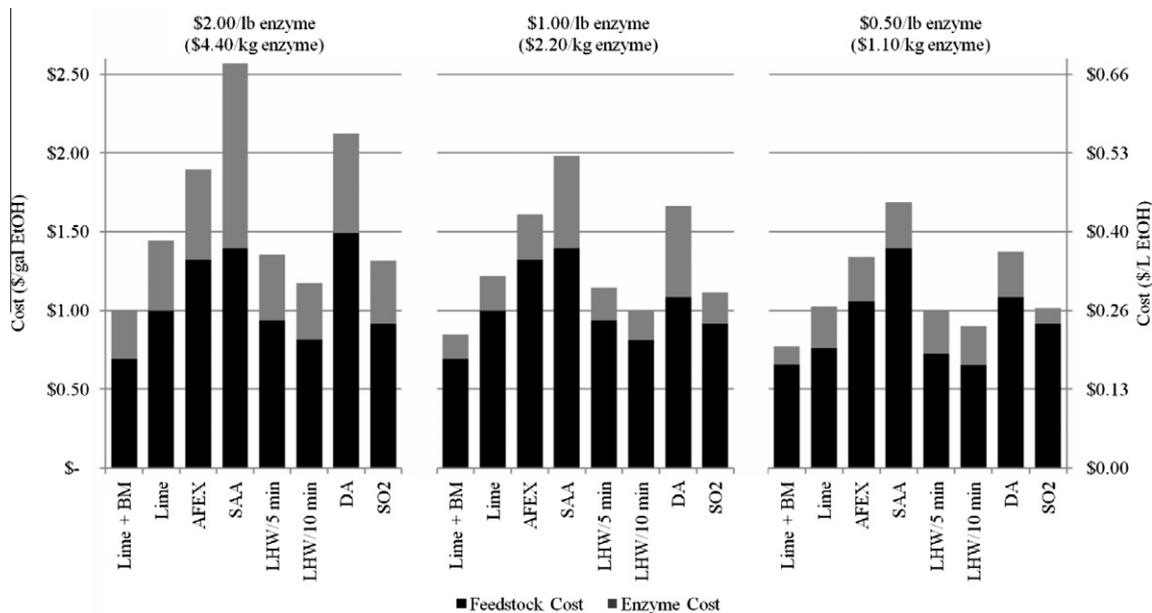


Fig. 4. Enzyme and feedstock cost contributions for three bulk enzyme costs assuming \$46/ton biomass.

Enzyme cost

$$\frac{\$4.41}{\text{kg enzyme}} \times \frac{\text{kg enzyme}}{92.3 \text{ kg digested sugar}} \times \frac{\text{kg digested sugar}}{0.51 \text{ kg EtOH}} \times \frac{1}{0.9} \times \frac{0.791 \text{ kg EtOH}}{\text{L EtOH}} = \frac{\$0.08}{\text{L EtOH}} = \frac{\$0.31}{\text{gal EtOH}}$$

Fig. 4 shows the calculated feedstock and enzyme costs for each pretreatment at the enzyme loading ratio that minimizes cost assuming \$46/ton biomass. Aden and Foust estimate that all other costs (pretreatment, utilities, labor, capital, etc.) should contribute approximately \$1.34/gal ethanol (current) or \$0.73/gal ethanol (goal). Eggeman and Elander have shown that there is little difference in cost between pretreatment technologies (Eggeman and Elander, 2005). In the study, the most cost-effective pretreatment (as measured by feedstock and enzyme costs alone) was lime + ball-milling with an estimated cost of \$2.55/gal ethanol (current) or \$1.73/gal ethanol (goal). However, this pretreatment used a costly mechanical process that was not considered in Aden and Foust's estimated pretreatment cost, so further economic analysis is required. This case is included to show the potential benefit of developing an economically feasible mechanical pretreatment technique. In the case of SO₂ treatment, which is similar to the pretreatment considered by Aden and Foust, the estimated cost is \$2.94/gal ethanol (current) or \$2.05/gal ethanol (goal). (Note: None of these cost estimates include credits for free sugars recovered in washing or pretreatment steps. When these sugars are included, costs will reduce accordingly.)

4.1. β -glucosidase effectiveness

Another purpose of this study was to determine the effectiveness of β -glucosidase addition when both cellulase and xylanase (5:1, 2:1, and 1:1) are used for hydrolysis. In the absence of β -glucosidase, the total enzyme loading was 30 mg protein/g raw glucan. With β -glucosidase addition, the total enzyme loading was 33.4 mg protein/g raw glucan. After a 72-h hydrolysis, overall carbohydrate yields were compared between the samples loaded with β -glucosidase and those without. The results are shown in Table 3.

Lime pretreatment showed the least benefit from adding β -glucosidase and overall yields were relatively unaffected. The opposite

Table 3

Comparison of overall yields with the addition of β -glucosidase. Yields reported as g glucan + xylan digested/100 g glucan + xylan loaded.

	Without β -glucosidase 30 mg protein/g raw glucan			With β -glucosidase 34.4 mg protein/g raw glucan		
	5:1	2:1	1:1	5:1	2:1	1:1
Lime + ball-mill	87.07	87.35	87.10	94.64	97.01	97.10
Lime	78.81	81.44	78.66	78.10	79.28	78.49
AFEX	44.96	45.72	45.59	49.33	50.24	50.42
SAA	65.04	65.49	67.29	68.16	69.46	71.18
LHW5 min	66.00	64.11	64.18	68.90	67.54	64.81
LHW10 min	73.20	70.29	66.40	76.42	74.50	74.02
DA	65.96	74.25	75.06	77.64	75.27	74.36
SO ₂	83.62	88.36	85.08	93.94	88.20	86.42

effect occurred with the lime + ball-milled pretreated sample. There was a dramatic increase in overall yield when β -glucosidase was added and there was a positive correlation with increased xylanase. The overall yield improved by 7.6% (5:1), 9.7% (2:1), and 10.0% (1:1).

The acidic pretreatments (SO₂, dilute-acid) achieved large gains in overall yield with β -glucosidase addition at the 5:1 ratio. Overall yield improved by 10.3% for the SO₂ pretreatment and 11.7% for the dilute acid pretreatment. For both pretreatments, the 2:1 and 1:1 samples showed little benefit from β -glucosidase addition.

Like the lime + ball-milled pretreatment, AFEX showed a positive relationship between β -glucosidase addition and an increased xylanase ratio with yield increases of 4.4% (5:1), 4.5% (2:1), and 4.8% (1:1). This relationship was also seen in the LHW 200 °C/10-min case with improved overall yields of 3.2% (5:1), 4.2% (2:1), and 7.6% (1:1). The LHW 200 °C/5-min and SSA pretreatments had modest increases in overall yield with β -glucosidase addition, which ranged from 3–4%.

5. Conclusions

In all pretreatment cases, xylanase addition improved xylan yield and in all but the dilute-acid case, overall yields improved as well. Another key observation is that the optimum enzyme mixture depends on the composition of the pretreated material. Pre-

treatments with lower xylan composition (SO₂, dilute-acid) had less benefit from xylanase addition. Although β -glucosidase typically is a small percentage of the overall enzyme mixture, in most cases it significantly improves overall yields. Enzymatic yield relates the mass of carbohydrates generated by enzymatic hydrolysis per mass of enzyme protein added and typically ranges from 10 to 90 kg of sugar digested/kg of protein.

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