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Oxidative Lime Pretreatment of Alamo Switchgrass

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Abstract Previous studies have shown that oxidative lime pretreatment is an effective delignification method that improves the enzymatic digestibility of many biomass feedstocks. The purpose of this work is to determine the recommended oxidative lime pretreatment conditions (reaction temperature, time, pressure, and lime loading) for Alamo switchgrass (*Panicum virgatum*). Enzymatic hydrolysis of glucan and xylan was used to determine the performance of the 52 studied pretreatment conditions. The recommended condition (110°C, 6.89 bar O_2, 240 min, 0.248 g $Ca(OH)_2$/g biomass) achieved glucan and xylan overall yields (grams of sugar hydrolyzed/100 g sugar in raw biomass, 15 filter paper units (FPU)/g raw glucan) of 85.9 and 52.2, respectively. In addition, some glucan oligomers (2.6 g glucan recovered/100 g glucan in raw biomass) and significant levels of xylan oligomers (26.0 g xylan recovered/100 g xylan in raw biomass) were recovered from the pretreatment liquor. Combining a decrystallization technique (ball milling) with oxidative lime pretreatment further improved the overall glucan yield to 90.0 (7 FPU/g raw glucan).

Keywords Switchgrass · Pretreatment · Lime · Enzymatic digestion

Introduction

In a recent technoeconomic analysis of a current enzymatic ethanol process, lignocellulose feedstock and biomass pretreatment were the largest contributors to process costs with estimates of 38% and 19%, respectively [1]. To maximize yields from lignocellulosic feedstocks requires highly effective biomass pretreatments.

Currently, ethanol is derived from food crops (e.g., corn, sugarcane). Rather than using food crops for ethanol production, it is advantageous to use lignocellulosic biomass for the following reasons: (1) more abundant, (2) high yields, (3) large variety, and (4) lower cost [2]. Sources of lignocellulosic biomass include energy crops, agricultural crop residues, and

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wastes (e.g., industrial, food, and municipal solids) [3, 4]. The main disadvantage of using lignocellulosic biomass is its inherent resistance to enzymatic hydrolysis.

Lignocellulosic biomass is primarily composed of three components: cellulose, hemicellulose, and lignin. Some barriers that limit lignocellulose digestibility include high lignin content, cellulose crystallinity, high degree of cellulose polymerization, low accessible surface area, small pore volume, and presence of acetyl groups on hemicellulose [5–7]. In a lignocellulose-to-ethanol production process, the role of biomass pretreatment is to remove these barriers to generate more digestible biomass.

Many chemical pretreatments have been employed to increase enzymatic digestion of lignocellulose. Previous studies showed that alkaline pretreatments are highly effective at removing lignin, which improves enzymatic digestibility by increasing cellulose accessibility [8]. Alkaline pretreatments have also demonstrated the ability to significantly remove acetyl groups from hemicellulose, which lowers steric hindrance of enzymes [9]. This study employed lime ($Ca(OH)_2$) as the alkaline agent because of its low cost, compatibility with oxidants, ease of recovery, and ease of use [10]. Lime pretreatment has previously been studied for corn stover [11–14], bagasse [15, 16], and poplar wood [17–20].

When choosing a lignocellulosic feedstock, it is important to consider cost, adaptability, yield, and input requirements. The US Department of Energy has chosen switchgrass (*Panicum virgatum*), a perennial, warm season prairie grass [21], as a model biomass feedstock. Switchgrass is highly adaptable and tolerant to draught and poor soils, which allows it to be grown in high yields on marginal lands [22, 23]. It requires low nutrient inputs and is highly resistant to pests, minimizing fertilizer, herbicide, and pesticide use. Switchgrass can be grown in most of the eastern two thirds of the US, as well as Mexico and Canada. Average yields of 13.4 Mg/(ha year) have been achieved [21, 24].

This work was performed in cooperation with the Consortium for Applied Fundamentals and Innovation (CAFI). In late 1999, the CAFI team was formed to include leaders in biomass pretreatment and hydrolysis. To compare the effectiveness of leading pretreatment technologies, the members observed a need to develop consistent methods [25, 26]. The CAFI team employs common feedstocks, shared enzymes, and identical analytical and reporting methods. CAFI 1 and CAFI 2 studied pretreatment of corn stover [27] and poplar wood [19]. This work was performed as part of CAFI 3, which focuses on increasing the enzymatic digestibility of switchgrass.

The primary goal of this work was to determine the effectiveness of oxidative lime pretreatment on Alamo switchgrass and to recommend the reaction time, pressure, temperature, and lime loading that produces the most enzymatically digestible switchgrass. This recommended condition was determined by considering pretreatment solid yield, pretreatment carbohydrate yield, and enzymatic yield. Furthermore, to examine the difference between each variety of switchgrass, the recommended treatment condition was compared to that obtained for Dacotah switchgrass in a previous study.

Materials and Methods

Substrate and Enzymes

The primary feedstock used in this study was the Alamo variety of switchgrass (*P. virgatum*). This variety is a southern lowland ecotype with thick stems. It was planted in Ardmore, OK on June 11, 2007 and harvested on November 11, 2007. During the growing season, total fertilizer applications were approximately 100.9 kg of nitrogen/ha and

50.4 kg of phosphorous/ha. Five small square bales were harvested and shipped from Ardmore, OK to Haven Research, Inc. (Golden, CO).

The second variety used in this study was Dacotah switchgrass. Dacotah is a northern upland switchgrass with thin stem morphology. It was planted in Pierre, SD on December 6, 1999 and harvested on March 1, 2008 after the plot stood over the winter. During the last growth season, no fertilizer or herbicide was utilized. Three small square bales were harvested and shipped from Pierre, SD to Hazen Research, Inc. (Golden, CO).

Once both varieties arrived at Hazen Research, Inc., the bales were shredded and then milled using a hammer mill equipped with a 1/4-in. screen. While keeping each variety separate, the combined milled materials were homogenized using the coning and quartering technique, separated into 5-kg sublots, and delivered to the Texas A&M Laboratory.

Both Alamo and Dacotah feedstocks were kindly provided by Ceres, Inc. (Thousand Oaks, CA). Their respective measured compositions are reported in Table 1.

Cellulase was Spezyme CP® (lot 301-04075-054, 82 mg protein/mL, 59 FPU/mL), which was kindly provided by Genencor®, a Danisco Division (Palo Alto, CA). The β-glucosidase was Novozyme 188® (67 mg protein/mL, 600 cellobiase units (CBU)/mL) and was obtained from Sigma-Aldrich (St. Louis, MO).

Pretreatment Methods

Substrate Preparation

Prior to pretreatment, the switchgrass was further milled to pass through 40 mesh (ASTM, West Conshohocken, PA) and prewashed in 200 g batches. Each batch 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.

Short-Term

Short-term 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

Table 1 Composition of raw switchgrass

Constituent	Dacotah (% dry weight)	Alamo (% dry weight)
Glucan	35.0	33.2
Xylan	21.8	21.0
Lignin	21.4	17.9
Arabanin	3.5	3.2
Sucrose	1.5	4.0
Acetyl	2.8	2.5
Protein	1.4	5.7
Extractives	8.1	10.2
Ash	3.3	3.7
Total	98.8	101.4

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-pressure 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 set at the desired reaction temperature. Initial heat-up time of the reaction contents was included in the overall reaction time. Upon completing the desired reaction time, reactors were removed from the oven and immediately placed in an ice bath to quench the reaction. 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 reaction contents underwent the post-pretreatment conditioning procedure.

Long-Term

Long-term pretreatment was conducted in plastic 450-mL bottles. The bottles were loaded with 16 g dry switchgrass each and excess calcium hydroxide (1 g $Ca(OH)_2$/g dry biomass). Water was added at a ratio of 15 g/g dry biomass. Compressed air was supplied through a manifold and bubbled into each bottle at 1.01 bar pressure. The bottles were placed in a temperature-controlled oven set at the reaction temperature of either 55 or 65°C. Stirring was performed manually twice per day using stainless steel spatulas. The water level of each bottle was checked regularly and additional water was added when necessary. Reaction time was 28 days, after which the post-pretreatment conditioning procedure was performed.

Post-Pretreatment Conditioning

The lime-treated biomass slurry was neutralized using 5 N HCl to a pH of approximately 4.0 to solubilize any residual lime 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.

Ball Milling

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.

Lime Consumption

As part of the post-pretreatment conditioning, the lime-treated biomass slurry was neutralized using 5 N HCl. The volume of 5 N HCl required to titrate the solution to an endpoint of pH 7.0 was recorded and used to calculate the amount of unreacted excess lime present in the pretreatment slurry. Using this value and the known initial quantity of lime, the amount of lime consumed was calculated.

Compositional Analysis

Compositional analysis was performed on the raw and pretreated samples. The material was prepared by air drying to a measured moisture content of <10%. The composition was analyzed using a National Renewable Energy Laboratory (NREL, Golden, CO) acid hydrolysis procedure [28]. 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 at 121°C 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 high-performance liquid chromatography (HPLC) analysis (Bio-Rad Aminex HPX-87P column, HPLC-grade water mobile phase, 0.6 mL/min, 80°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.

Sugar Analysis in the Pretreatment Liquor

Prior to neutralizing the lime-treated biomass slurry, a 10-mL aliquot of pretreatment liquor was obtained using vacuum filtration. The monomeric sugar content of the pretreatment liquor was quantified using HPLC analysis (Bio-Rad Aminex HPX-87P column, HPLC-grade water mobile phase, 0.6 mL/min, 80°C column temperature).

The oligomeric sugar content of the pretreatment liquor was quantified by subjecting the pretreatment liquor to acid hydrolysis with 4% sulfuric acid using an autoclave at 121°C for 1 h. HPLC analysis was used to measure the glucose and xylose concentrations of each sample, which were then recalculated as equivalent glucan and xylan concentrations.

Enzymatic Hydrolysis

The enzymatic hydrolysis procedure for both glucan and xylan closely followed the enzymatic saccharification procedure provided by NREL [29]. Hydrolysis samples were prepared in 50-mL plastic centrifuge tubes. Pretreated biomass loading weight was calculated based on moisture content and glucan 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), 0.04 mL cycloheximine (10 mg/mL in distilled water), 1 mL of each enzyme dilution (cellulase, 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 a desired enzyme loading. The cellulase enzyme loading was 15 FPU/g raw glucan, and β-glucosidase was loaded in excess at a loading of 60 CBU/g raw glucan. Hydrolysis occurred in a shaking incubator (100 rpm) at 50°C for 72 h. To quench the hydrolysis, the samples were placed in a 105°C oven for 5 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°C column temperature) was used to measure the glucose and xylose concentrations of each

sample. These concentrations were then recalculated as equivalent glucan and xylan concentrations to report digestibility yields.

Experimental Design

The primary goal of this work was to assess the effectiveness of oxidative lime pretreatment in increasing the enzymatic digestibility of Alamo switchgrass. A total of 52 different pretreatments (Table 2) were performed using a full-factorial experimental design of five temperatures (100, 110, 120, 140, and 150°C), three O_2 pressures (3.45, 6.89, and 10.3 bar absolute O_2), and four reaction times (60, 120, 180, and 240 min). Because of the severe conditions, the high-pressure pretreatments (10.3 bar O_2) were only run at 100, 110, and 120°C. The recommended pretreatment condition (reaction time, lime loading, temperature, and O_2 pressure) was determined by considering pretreatment yield, carbohydrate yield, and enzymatic yield. The long-term reactions involving both Alamo and Dacotah switchgrass were conducted at reaction temperatures of 55 and 65°C, reaction pressure of 1.01 bar O_2, and reaction time of 28 days. Overall enzymatic yields were obtained using a 72-h enzymatic hydrolysis with a cellulase loading of 15 FPU/g glucan in raw biomass and an excess β-glucosidase loading of 60 CBU/g glucan in raw biomass.

Results and Discussion

Lime Consumption

Figure 1 shows lime consumption as a function of lignin removed. Lime consumption ranged from 0.074 to 0.375 g lime consumed/g raw biomass (Table 3). At the recommended pretreatment condition (110°C, 6.89 bar O_2, 240 min), lime consumption was 0.248 g lime consumed/g raw biomass.

Sugars Recovered from Pretreatment Liquor

When analyzing pretreatment carbohydrate yields, it is important to note that significant amounts of carbohydrates can be solubilized during the oxidative lime pretreatment process. Analysis of the pretreatment liquor revealed very low concentrations of monomeric glucose or xylose; however, glucan and xylan oligomers were present in more moderate concentrations. Table 4 shows the concentrations of glucan and xylan oligomers recovered in the pretreatment liquor for several representative pretreatment conditions. Out of the five samples reported, only one condition (150°C, 3.45 bar O_2, 240 min) contained a significant amount of glucan (8.1 g glucan recovered/100 g glucan in raw). Substantial xylan

Table 2 Short-term pretreatment conditions

Pressure (bar O_2)	Time (min)	Temperature (°C)
3.45	60, 120, 180, 240	100, 110, 120, 140, 150
6.89	60, 120, 180, 240	100, 110, 120, 140, 150
10.3	60, 120, 180, 240	100, 110, 120

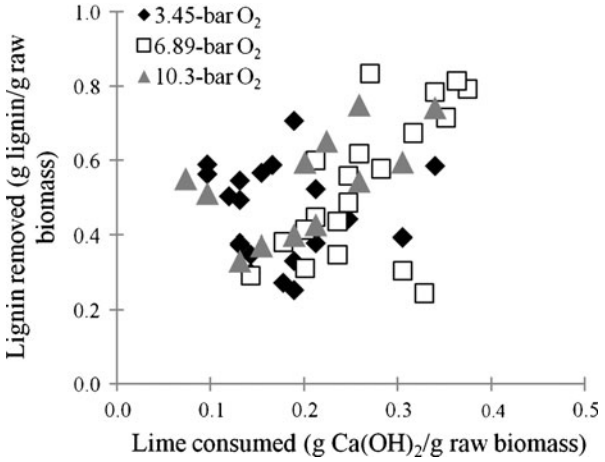

Fig. 1 Correlation of lignin removal with lime consumption

oligomers were recovered in all five samples with xylan recoveries of 23.7 g (120°C, 3.45 bar O_2, 240 min), 25.2 g (150°C, 3.45 bar O_2, 240 min), 25.7 g (140°C, 6.89 bar O_2, 120 min), 26.0 g (110°C, 6.89 bar O_2, 240 min), and 27.4 g (150°C, 6.89 bar O_2, 240 min) xylan recovered/100 g xylan in raw.

Pretreatment Yields

The primary goal of oxidative lime pretreatment is to remove lignin, while minimizing glucan and xylan degradation. When comparing pretreatment effectiveness, it is important to consider the degradation of each of these three key components. As pretreatment severity increases, more lignin is removed at the sacrifice of glucan pretreatment yields. This portion of the work focused on finding a balance between glucan recovery and lignin removal.

Table 3 Lime consumption (grams of $Ca(OH)_2$/gram of raw biomass)

Time (min)	100°C	110°C	120°C	140°C	150°C
3.45 bar O_2					
60	0.18	0.19	0.14	0.19	0.21
120	0.14	0.13	0.13	0.12	0.17
180	0.31	0.25	0.21	0.10	0.15
240	0.13	0.13	0.34	0.10	0.19
6.89 bar O_2					
60	0.33	0.14	0.31	0.20	0.24
120	0.24	0.18	0.20	0.28	0.35
180	0.25	0.21	0.26	0.34	0.37
240	0.21	0.25	0.32	0.27	0.36
10.3 bar O_2					
60	0.13	0.19	0.15		
120	0.21	0.26	0.31		
180	0.10	0.20	0.34		
240	0.07	0.22	0.26		

Table 4 Sugars recovered from pretreatment liquor

Pretreatment conditions			Sugars recovered[a]	
Temperature (°C)	Pressure (bar O_2)	Time (min)	Glucan	Xylan
120	3.45	240	3.27	23.67
150	3.45	240	8.11	25.15
110	6.89	240	2.62	26.03
140	6.89	120	5.41	25.65
150	6.89	240	2.44	27.42

[a] Grams of component recovered/100 g component in raw biomass

Pretreatment yields of the solid material (Fig. 2) were calculated using the following definition:

$$Y_i = \frac{C_i Y_t}{C_{i0}}$$

where:

i component (lignin L, glucan G, xylan X)

Y_i pretreatment yield of component i at time t (grams of residual component i/gram of component i in raw biomass)

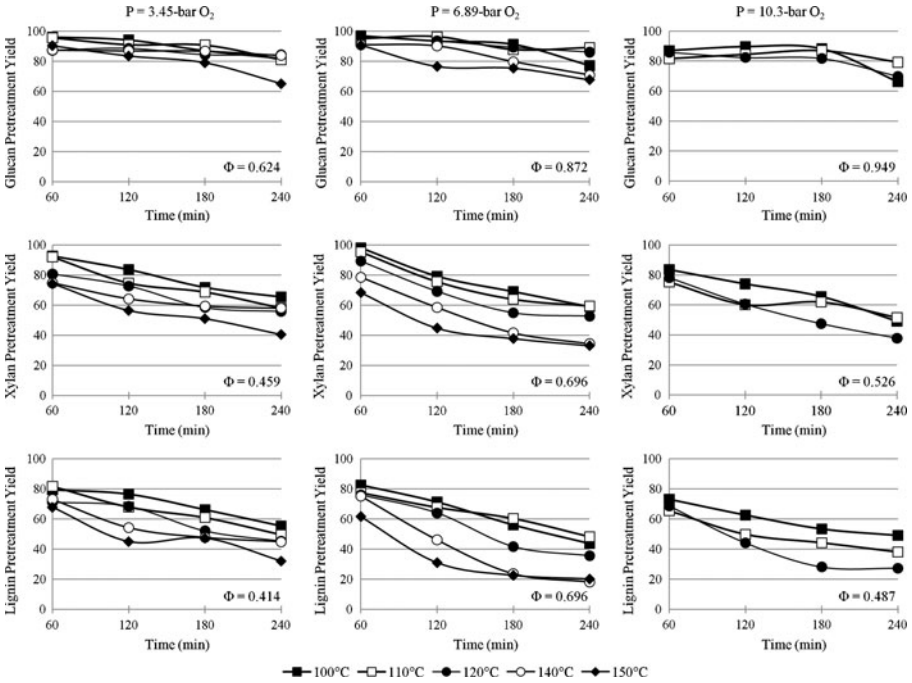

Fig. 2 Short-term pretreatment yields. Φ is the average standard deviation (all pretreatment yields are expressed as grams of component recovered/100 g component in raw biomass)

C_{i0} component i content at time 0 (grams of component i in raw biomass/gram of raw biomass)
C_i component i in time t (grams of residual component i/gram of residual biomass)
Y_t total solids pretreatment yield at time t (grams of residual biomass/gram of raw biomass)

Glucan Pretreatment Yields

The pretreatments performed at 3.45 bar O_2 were the most successful in maintaining high glucan pretreatment yields (grams of glucan recovered/100 g glucan in raw biomass; Fig. 2). High glucan recoveries of 96.0 (100°C, 60 min) and 95.6 (110°C, 60 min) were observed. Increased reaction temperature and time led to the lowest glucan yields of 78.9 (140°C, 240 min) and 65.1 (150°C, 240 min), with the remaining glucan pretreatment yields >80.

Increased pressure (6.89 bar O_2) had little effect on glucan pretreatment yields. At this pressure, four conditions resulted in glucan pretreatment yields <80 with 67.6 (150°C, 240 min) and 70.8 (140°C, 240 min) being the lowest observed. The majority of glucan pretreatment yields at this pressure ranged from 80 to 95, with the maximum being 97.0 (100°C, 60 min).

At the highest pressure (10.3 bar O_2), glucan pretreatment yields began to decline. Of the 12 pretreatments performed, three resulted in glucan pretreatment yields below 80, with 66.2 (100°C, 240 min) as the minimum. The highest yields observed were 89.7 (100°C. 120 min) and 88.2 (180 min). By studying the average glucan pretreatment yield for each pressure (86.2, 3.45 bar O_2; 86.6, 6.89 bar O_2; 81.9, 10.3 bar O_2), it is clear that 6.89 bar O_2 is the ideal pressure for achieving high glucan recovery.

Xylan Pretreatment Yields

As expected, xylan pretreatment yields (grams of xylan recovered/100 g xylan in raw biomass; Fig. 2) were highest in the pretreatments performed at the lowest pressure (3.45 bar O_2). At this pressure, very high pretreatment xylan yields of 92.6 (100°C, 60 min) and 91.9 (110°C, 60 min) were observed. At more severe reaction temperatures and longer times, xylan pretreatment yields fell to 40.5 (150°C, 240 min) and 51.0 (150°C, 180 min).

As was the case with glucan pretreatment yields, increasing the pressure (6.89 bar O_2) did not significantly affect xylan pretreatment yields. Maximum yields of 98.1 (100°C, 60 min) and 95.4 (110°C, 60 min) and minimum yields of 34.5 (140°C, 240 min) and 33.1 (150°C, 240 min) were observed.

Further increasing the pressure to 10.3 bar O_2 reduced the number of samples with very high xylan pretreatment yields. The two maximum xylan pretreatment yields observed at this pressure were 83.7 (100°C, 60 min) and 78.3 (120°C, 60 min). Similar minimum yields as the 3.45- and 6.89-bar O_2 pretreatments were observed with values of 47.6 (150, 180 min) and 37.9 (150, 240 min).

As discussed in the "Sugars Recovered from Pretreatment Liquor" section, oxidative lime pretreatment solubilizes a significant portion of the xylan in switchgrass, resulting in lower xylan pretreatment yields. Average xylan pretreatment yields were 67.7, 63.1, and 62.2 for the 3.45-, 6.89-, and10.3-bar O_2 cases, respectively. This showed there was only a slight decline in xylan pretreatment yields because of increased pressure; however, for each pressure, there was a large range in xylan pretreatment yields and increased reaction time

dramatically reduced xylan pretreatment yields. One particular case (140°C, 6.89 bar O_2) showed a decline of 44.1 percentage points by increasing the reaction time from 60 to 240 min.

Lignin Pretreatment Yields

As stated previously, the primary purpose of oxidative lime pretreatment is to remove lignin, thus low lignin pretreatment yields (grams of lignin recovered/100 g lignin in raw biomass; Fig. 2) are desired. In this work, there was a strong positive correlation between lignin removal and increased enzymatic digestibility. The pretreatments performed at 3.45 bar O_2 were the least successful in removing lignin. Low temperatures and short reaction times produced the highest lignin yields of 79.6 (100°C, 60 min) and 81.7 (110°C, 60 min). The lowest lignin pretreatment yields observed were 32.2 (150°C, 240 min) and 45.0 (140°C, 240 min).

Increasing reaction pressure to 6.89 bar O_2significantly reduced lignin pretreatment yields; however, increased pressure could not compensate for low temperature and short reaction times. High lignin pretreatment yields of 82.6 (100°C, 60 min) and 77.5 (110°C, 60 min) were still observed at these mild conditions. Increasing the severity at this pressure did result in the lowest observed lignin pretreatment yields, with four pretreatments achieving lignin pretreatment yields below 23. The lowest lignin pretreatment yields were 18.3 (140°C, 240 min) and 20.3 (150°C, 240 min).

At the highest pressure (10.3 bar O_2), the maximum lignin pretreatment yields were 73.2 (100°C, 60 min) and 68.7 (120°C, 60 min). Two successful pretreatments that obtained lignin pretreatment yields below 30 were 28.3 (120°C, 180 min) and 27.4 (120°C, 240 min).

Overall, selected pretreatment conditions could remove lignin. It was clear that reaction times of 180 or 240 min were required to significantly remove lignin. The pretreatments performed at the lowest pressure (3.45 bar O_2) were the least promising, with an average lignin pretreatment yield of 59.5. Increased pressure clearly improved lignin removal with average lignin pretreatment yields of 51.2 (6.89 bar O_2) and 50.4 (10.3 bar O_2).

Pretreatment Yield Summary and Recommended Condition

In general, oxidative lime pretreatment successfully removed lignin while maintaining high recoveries of glucan. Significant xylan degradation was observed, but it never exceeded lignin degradation, and a large concentration of xylan oligomers can be recovered from the pretreatment liquor.

The recommended conditions for oxidative lime pretreatment of Alamo switchgrass (110°C, 6.89 bar O_2, 240 min) obtained a glucan pretreatment yield of 89.0 g glucan recovered/100 g glucan in raw biomass, xylan pretreatment yield of 59.4 g xylan recovered/100 g xylan in raw biomass, and a lignin pretreatment yield of 48.2 g lignin recovered/100 g lignin in raw biomass. If the glucan and xylan oligomers are recovered from the pretreatment liquor, glucan pretreatment yield improves to 91.6 g glucan recovered/100 g glucan in raw biomass and xylan pretreatment yield improves to 85.4 g xylan recovered/100 g xylan in raw biomass.

Enzymatic Yields

The primary goal of biomass pretreatment is to increase the enzymatic digestibility of lignocellulosic biomass. When comparing pretreatment performance, it is important to

measure the enzymatic digestibility of both glucan and xylan, while considering glucan and xylan pretreatment yields. This study used a 72-h hydrolysis with a cellulase loading of 15 FPU/g raw glucan and an excess β-glucosidase loading of 60 CBU/g raw glucan. When choosing a recommended pretreatment condition (reaction temperature, time, pressure, and lime loading),the determining factor was overall yield of glucan and xylan. Overall yield Y_{oi}) is defined as the amount of glucan or xylan enzymatically hydrolyzed after pretreatment per unit of glucan or xylan in the raw feedstock.

$$Y_{oi} = Y_i \times Y_{ei}$$

where:

i component (glucan G or xylan X)
Y_{oi} overall yield of component i (grams of hydrolyzed component i/gram of component i in raw biomass)
Y_i pretreatment yield of component i (grams of residual component i/gram of component i in raw biomass)
Y_{ei} enzymatic yield of component i (grams of hydrolyzed component i/gram of component i in pretreated biomass)

Enzymatic hydrolysis results are shown in Fig. 3. Pretreatments performed at the lowest pressure (3.45 bar O_2) were the least successful in producing highly digestible switchgrass. As discussed in the "Pretreatment Yields" section, although glucan recovery after pretreatment was quite high for this set of pretreatments, overall lignin removal was not substantial. With high lignin contents remaining in the pretreated biomass, overall glucan yields were low (grams of glucan hydrolyzed/100 g glucan in raw biomass) and generally ranged from 55 to 65. The highest overall glucan yields observed were 66.6 (120°C, 240 min) and 66.4 (100°C, 60 min). The worst performing condition had an overall glucan yield of just 46.9 (150°C, 240 min), well below the average overall glucan yield (58.9) for the pretreatments performed at this pressure. Xylan overall yields (grams of xylan hydrolyzed/100 g xylan in raw biomass) were moderate as well, typically in the range of 40–45. The maximum and minimum overall xylan yields were 55.8 (100°C, 60 min) and 27.6 (150°C, 240 min), respectively.

A good balance between glucan recovery and lignin removal was demonstrated in the pretreatments performed at 6.89 bar O_2. This balance resulted in a significant positive shift in overall glucan yields. At this pressure, the recommended pretreatment condition (110°C, 6.89 bar O_2, 240 min) produced an overall glucan yield of 85.9 and an overall xylan yield of 52.2. In terms of overall glucan yield, several other successful pretreatments resulted in high overall glucan yields of 81.9 (140°C, 120 min), 80.3 (120°C, 180 min), and 79.5 (140°C, 180 min). The lowest overall glucan yield was 63.9 (150°C, 180 min). The average overall glucan yield for pretreatments at this pressure was 73.2, considerably higher than the average observed for the low-pressure pretreatments (3.45 bar O_2). Overall xylan yields were only slightly improved over the 3.45-bar O_2 pretreatments, with yields primarily ranging from 40 to 50. The highest overall xylan yield observed at this pressure was 53.5 (100°C, 60 min), whereas the lowest was 31.1 (150°C, 240 min).

At the highest pressure (10.3 bar O_2), significant glucan degradation occurred during pretreatment, which reduced overall glucan yields. For the high-pressure pretreatments, the maximum overall glucan yields were 77.0 (120°C, 180 min) and 76.2 (110°C, 240 min). The least successful pretreatment at this pressure produced an overall glucan yield of 59.1 (100°C, 60 min). The average overall glucan yield for the 6.89-bar O_2 pretreatments was 68.4, which was between the 3.45- and 6.89-bar O_2 conditions. Similar to the other

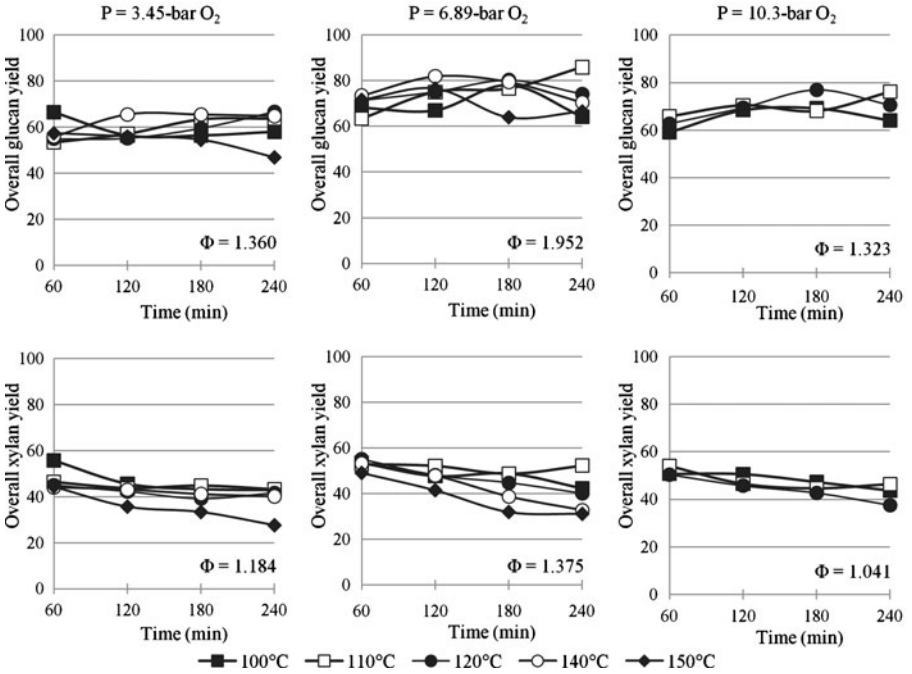

Fig. 3 Overall enzymatic yield results for short-term pretreatments. Enzymatic hydrolysis was performed for 72 h with a cellulase enzyme loading of 15 FPU/g glucan in raw biomass. *Φ* is the average standard deviation (all overall enzymatic yields are expressed as grams of component hydrolyzed/100 g raw component)

pressures, overall xylan yields were moderate, typically 45–50. The maximum and minimum overall yields observed were 54.0 (110°C, 60 min) and 37.5 (120°C, 240 min), respectively.

Alamo and Dacotah Comparisons

Another purpose of this study was to compare the enzymatic digestibility of lime-pretreated Alamo switchgrass with lime-pretreated Dacotah switchgrass. Alamo switchgrass is a southern lowland variety, whereas Dacotah is a northern upland variety. In terms of morphology, Alamo is thick-stemmed and Dacotah is thin-stemmed. The latitude of origin was quite different as well for the two varieties, with Alamo (29° N) being much further south than Dacotah (46° N). The Alamo variety was harvested from Ardmore, OK (34° N), and Dacotah from Pierre, SD (44° N), both close to their latitude of origin. Alamo was harvested in late fall of the same year it was planted, whereas Dacotah stood over the winter before harvesting. The differences in ecotype, morphology, harvest location, and harvest date resulted in compositional differences that altered the recommended conditions of oxidative lime pretreatment.

Long-Term Comparison

The first comparison performed was a long-term lime pretreatment of the two varieties. This pretreatment was conducted at 55°C over 28 days, with compressed air bubbled into

the reaction bottles. Enzymatic digestibility (Fig. 4) was measured using a 72-h hydrolysis time with a cellulase loading of 15 FPU/g raw glucan and an excess β-glucosidase loading of 60 CBU/g raw glucan.

On a treated glucan basis (grams of glucan hydrolyzed/100 g pretreated glucan), Alamo was significantly more digestible (82.2) than Dacotah (58.5). However, overall glucan yields (grams of glucan/100 g glucan in raw), which factor in glucan pretreatment yields, were much more similar. Alamo and Dacotah had overall glucan yields of 60.0 and 53.2, respectively. Xylan enzymatic yields followed a similar trend. On a treated basis, Alamo was 10.5 percentage points more digestible than Alamo, but only 3.3 percentage points more digestible on an overall basis.

Recommended Pretreatment Conditions

There are key differences in the genotype (lowland vs. upland), ecotype (southern vs. northern), morphology, and harvest dates of the Alamo and Dacotah samples used in this work. Alamo is a southern lowland switchgrass with thick-stem morphology and a late fall harvest date. Dacotah is a northern upland, thin-stemmed variety that was harvested in the late spring after standing over the winter. Holocellulose content generally increases with latitude for upland varieties, whereas the opposite is true for lowland varieties [30, 31]. The Alamo used in this study was harvested 5° N of its latitude of origin, resulting in low cellulose content. Harvest time also probably affected Dacotah's higher cellulose content. It has been observed that harvesting in the spring after the switchgrass stood over the winter decreases mineral concentration but increases lignin and cellulose content [32, 33].

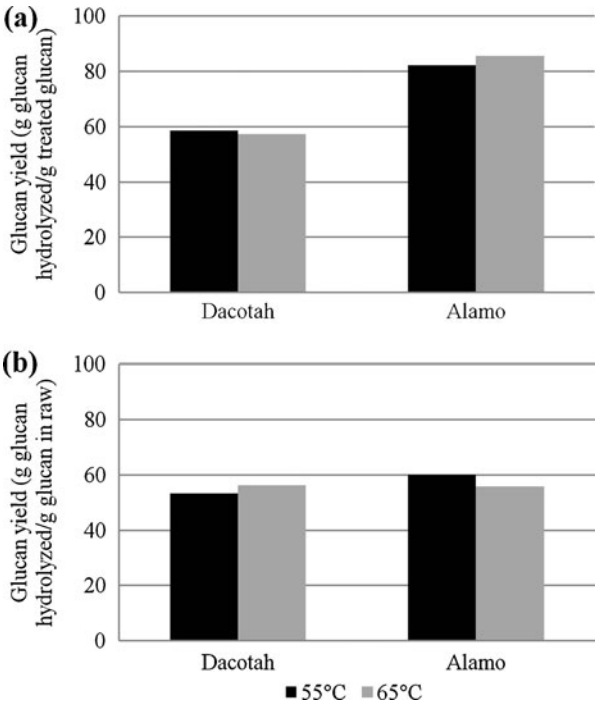

Fig. 4 Enzymatic yield results for long-term lime-pretreated Dacotah and Alamo switchgrass. Enzymatic hydrolysis was performed for 72 h with a cellulose enzyme loading of 15 FPU/g glucan in raw biomass. **a** Glucan yields on a treated basis, **b** glucan yields on a raw basis

Although Dacotah had more cellulose content, Alamo had significantly lower lignin. These compositional differences alter how the switchgrass responds to oxidative lime pretreatment.

In a previous study, the recommended pretreatment condition for Dacotah switchgrass was 120°C, 6.89 bar O_2, 240 min [34]. After pretreatment at these conditions, Dacotah switchgrass had an overall glucan yield (grams of glucan hydrolyzed/100 g of glucan in raw biomass) of 85.2 and an overall xylan yield (grams of xylan hydrolyzed/100 g xylan in raw biomass) of 50.1. This was quite similar to the maximum glucan (85.9) and xylan (52.2) overall yields reported for Alamo in this study. Recommended pretreatment time and pressure were identical for the two varieties; however, the pretreatment temperature was 10°C less for Alamo. The less severe temperature most likely results from the lower lignin content in the Alamo variety. In general, the Alamo switchgrass was more digestible on a treated basis (grams of glucan hydrolyzed/100 g pretreated glucan), but suffered from low pretreatment solids yield. At their respective recommended pretreatment conditions, Dacotah had a pretreatment solids yield of 72.0, whereas Alamo was significantly lower (63.7).

For each variety, another useful metric is to compare how oxidative lime pretreatment selectively removed lignin compared to xylan. Figure 5 clearly reveals that oxidative lime pretreatment selectively removed more lignin from Dacotah (1.32 g lignin/g xylan) compared to Alamo (1.16 g lignin/g xylan).

Ball Milling Comparison

The "Pretreatment Yields" section demonstrated the effectiveness of oxidative lime pretreatment as a delignification technique, which improved overall sugar yields ("Enzymatic Yields" section). Another key barrier to enzymatic digestion of lignocellulose is cellulose crystallinity. Ball milling is a laboratory decrystallization technique that can be used in conjunction with oxidative lime pretreatment. Although not economical at the industrial scale, ball milling can be used to demonstrate the benefit of combining chemical and mechanical pretreatment methods. By lowering lignin content and cellulose crystallinity, high overall yields can be achieved with reduced enzyme loadings.

Compared to oxidative lime pretreatment alone, adding ball milling achieved slightly higher overall glucan but at a much lower cellulase loading (Table 5). Combining pretreatment techniques to Alamo switchgrass (110°C, 6.89 bar O_2, 240 min, 72-h ball milling) produced an overall glucan yield of 90.0 at a cellulase loading of 7 FPU/g raw glucan. At the same enzyme loading, Dacotah switchgrass (120°C, 6.89 bar O_2, 240 min, 72-h ball milling) obtained an overall glucan yield of 91.1. Xylan overall yields were 47.0 and 42.4 for the Alamo and Dacotah varieties, respectively.

Factoring in easily digestible sugars and oligomers recovered from the pretreatment liquor dramatically improves overall yields. Including sugars and oligomers from the pretreatment liquor, Alamo achieved an overall glucan yield of 92.6 and an overall xylan yield of 73.0. Similarly, Dacotah achieved overall glucan and overall xylan yields of 92.9 and 67.6, respectively.

Conclusions

This work demonstrates that oxidative lime pretreatment significantly increases enzymatic digestibility of Alamo switchgrass. At the recommended condition (110°C, 6.89 bar O_2,

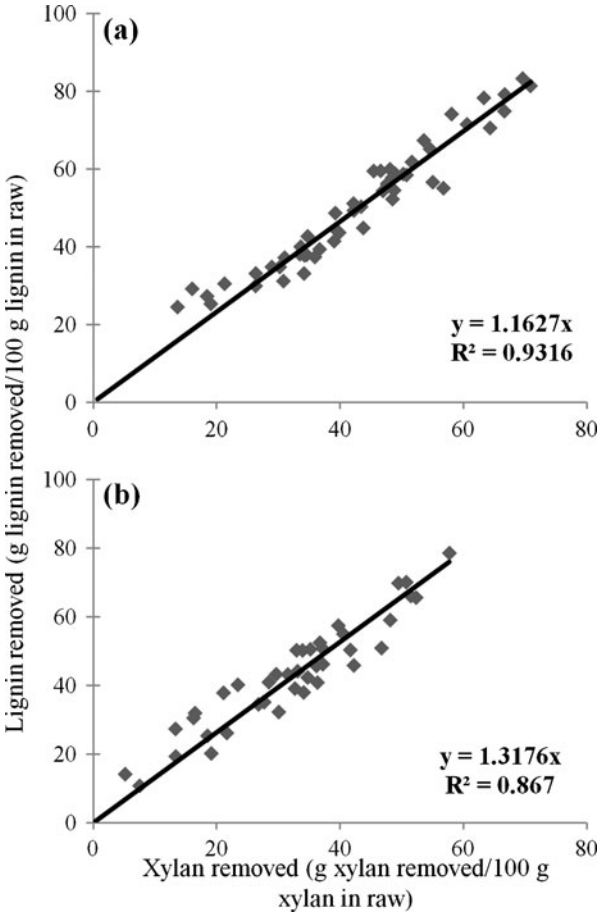

Fig. 5 Selectivity as a function of lignin and lime removal. **a** Short-term lime pretreatment of Alamo switchgrass, **b** short-term lime pretreatment of Dacotah switchgrass

240 min), overall glucan and xylan yields (grams of sugar hydrolyzed/100 g sugar in raw biomass; 15 FPU/g raw glucan) were 88.5 and 78.2, respectively, when sugars and oligomers from the pretreatment liquor are included. With the addition of ball milling to oxidative lime pretreatment, overall glucan and xylan yields (including sugars and

Table 5 Overall digestibility of oxidative lime-treated and ball-milled switchgrass

Variety	Enzymatic yields (g component hydrolyzed/100 g component in raw biomass)		Sugars recovered from pretreatment liquor (g component solubilized/100 g component in raw biomass)		Overall digestibility (g component hydrolyzed/100 g component in raw biomass)	
	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan
Alamo[a]	90.0	47.0	2.6	26.0	92.6	73.0
Dacotah[b]	91.1	42.4	1.8	25.2	92.9	67.6

Includes sugars digested from pretreated solids and oligomeric sugars from pretreatment liquor

[a] 110°C, 6.89 bar O_2, 240 min, 72-h ball milling

[b] 120°C, 6.89 bar O_2, 240 min, 72-h ball milling

oligomers in the pretreatment liquor) improved to 92.9and 67.6, respectively, with a lower enzyme loading (7 FPU/g raw glucan). When compared to Dacotah switchgrass, Alamo had lower pretreatment solid yields, but still achieved similar glucan digestibility with a slight decrease in reaction temperature (10°C).

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