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ARTICLE *in* ENERGY & FUELS · JULY 2009

Impact Factor: 2.79 · DOI: 10.1021/ef900043z

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Dilute Acid Pretreatment of Oven-dried Switchgrass Germplasms for Bioethanol Production†

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Received January 16, 2009. Revised Manuscript Received May 6, 2009

Bioethanol production potential of three oven-dried switchgrass germplasms (St6−1, St6−3E, and St6−3F) containing 26.65−29.28% glucan, 17.92−19.37% xylan, and 17.74−19.23% lignin (dry matter basis) was investigated. Evaluation of the effect of three acid concentrations (0.5, 1.0, and 1.5% w/v) and residence times (30, 45, and 60 min) on composition of all germplasms indicated significant hemicellulose solublization relying greatly on pretreatment intensity. No apparent delignification was observed during pretreatment. Pretreated samples with the least lignin content or greatest hemicellulose solubilization within each germplasm were selected for hydrolysis and fermentation. Enzymatic hydrolysis at cellulase activities of 0, 15, and 30 FPU (filter paper units)/g dry biomass indicated that addition of cellulase significantly improved glucan hydrolysis ($P < 0.05$) but supplementation with xylanase did not statistically improve hydrolysis ($P > 0.05$). Glucan-to-glucose conversion was enhanced by acid pretreatments, especially those resulting in greater hemicellulose solubilization. The greatest glucan conversion of 91.8% was obtained from 60 min/1.5% acid pretreated St6−3E switchgrass hydrolyzed at 30 FPU cellulase/g dry biomass supplemented with xylanase. Fermentation of hydrolyzates by *Saccharomyces cerevisiae* (ATCC 24859) resulted in nearly complete utilization of glucose. The highest ethanol yield of 0.082 g ethanol/g raw St6−3E switchgrass corresponded with 53.5% of theoretical yield based on glucose fermentation. These results demonstrate that the new switchgrass germplasms are potential energy crops for bioethanol production through appropriate processing.

Introduction

The incessant increase in world energy consumption and concerns over diminishing fossil fuels have resulted in an urgent need for alternative energy sources that can sustainably power the world. Biomass is a competitive candidate capable of producing significant amounts of renewable energy while maintaining cost effectiveness through process optimization. Polysaccharides in biomass, either starch or cellulose, can be broken down to monosaccharides and fermented to produce bioethanol.[1]

Rapid growth in ethanol production has been witnessed over the past several years with the yield increasing from less than 11 billion liters in 2003 to more than 22 billion liters in 2007.[2] This upward trend is projected to continue and exceed 45 billion liters by 2010. Currently in the US, commercial production of bioethanol relies primarily on corn grain grown predominantly in the Midwest. Although the contribution of bioethanol to the overall gasoline market is small, enormous growth in ethanol production is believed to significantly impact the current corn market's structure, while promoting development of bioethanol processes from alternative biomass feedstocks.[2,3]

Lignocellulose, a major component of the plant cell wall, is an abundant biomass resource that has shown tremendous potential as a substrate for bioethanol production. However, conversion of lignocelluloses to ethanol is more challenging than the corn-to-ethanol process because of the complex structure of plant cell walls. Besides general saccharification and fermentation, a pretreatment step is needed to make the cellulose matrix more susceptible to cellulolytic enzymes during hydrolysis. Pretreatment is achieved by breaking the lignin seal and hemicellulose sheathing over cellulose and by disrupting the crystalline structure of cellulose.[4] Over the years, numerous pretreatment methods involving physical, chemical, and biological mechanisms have been studied. Dilute acid pretreatment is a widely explored chemical approach because of its ease of availability and effectiveness in hemicellulose solubilization, which opens up the overall lignin−polysaccharides network to facilitate subsequent cellulose conversion.[5,6]

A variety of lignocellulosic feedstocks such as forestry or agricultural residues, organic components of municipal and

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10.1021/ef900043z CCC: $40.75
Published on Web 06/09/2009

industrial wastes, and energy crops are being investigated for bioethanol production.[7] Switchgrass is an energy crop that has recently received considerable attention.[8,9] Studies have shown its potential for bioethanol production by dilute acid pretreatment and enzymatic saccharification. Dien et al.[10] reported that maximum monomeric sugar yields appeared to level off at a sulfuric acid concentration of 1.25% (w/v) when treating switchgrass at 10% solid loading in an autoclave at 121 °C for 60 min. Xylan, the major component of hemicellulose in switchgrass, could be dissolved within the first minute of pretreatment with 0.9% (w/w) sulfuric acid at a temperature of 180 °C.[11]

Although extensive research has been conducted on dilute sulfuric acid pretreatment of various lignocellulosic feedstocks including switchgrass, there is a need to further understand the impact of reaction conditions such as acid concentration and treatment time on specific grass germplasms. In addition, the primary focus of many previous studies on dilute acid pretreatment has been the optimization of monomeric sugar yield during hydrolysis and not necessarily the estimation of the final ethanol yield from fermentation. Fermentation is crucial in determining the potential inhibitory effects of acid pretreatment and is key for evaluating the overall process of cellulosic ethanol production.

Hence, this study investigated the varying effects of dilute sulfuric acid pretreatment on fermentability of three oven-dried switchgrass germplasms to ethanol as impacted by variations in composition. Hemicellulose solubilization and lignin degradation in three oven-dried switchgrass germplasms was studied. The impact of enzyme loading during hydrolysis with cellulase, cellobiase, and xylanase was also investigated. Fermentation with *Saccharomyces cerevisiae* was performed to estimate the overall ethanol yield potential of switchgrass as impacted by variety.

Experimental Section

Switchgrass Feedstock. Three switchgrass germplasms, well adapted to the Southeastern US, were obtained from the Central Crops Research Station, near Clayton, NC for this study. Each switchgrass field was a well established stand representing one of the three germplasms designated as St6−1, St6−3E, and St6−3F. Since sampling, St6−3E has been released under the name of "BoMaster" and St6−3F as "Performer".[12,13] Germplasms St6−1 and St6−3E were selected for high dry matter yield; whereas St6−3F, preferable as cattle feed, was selected for improved digestibility. Digestibility and dry matter yield are normally not positively associated;[13] however, at the time of sampling for this study, all three germplasms provided comparable dry matter yield of approximately 13 450 kg/ha (12 000 lbs/acre).

Switchgrass germplasms were harvested to about a 6 in. stubble on July 30, 2007. Three bulk samples, each representing one specific germplasm, were transported to a field laboratory wrapped in cloth sheets, where they were dried in cloth bags in a forced-air oven at 70 °C until constant weight was obtained (at least 3 days). Dried samples were ground to pass a 2 mm screen through a Wiley mill and stored in zip-locked plastic bags at room temperature at the Biological and Agricultural Engineering Department at NCSU, Raleigh, NC.

Dilute Acid Pretreatment. Sulfuric acid at 0.5, 1.0, and 1.5% (w/v) was used to pretreate oven-dried switchgrass germplasms at a solid-to-liquid ratio of 1:10 prepared with 3.0 g of grass samples in 100 mL serum bottles. The mixtures were autoclaved at 121 °C/15 psi under standard liquid cycle for residence times of 30, 45, and 60 min. Pretreatment intensity was maintained at low levels in this study to potentially reduce sugar degradation, formation of inhibitors like furfurals, and also operating costs in scaled up operations. Pretreated solids recovered by filtration through a porcelain Buchner funnel were washed with 250 mL hot deionized water to remove residual acid. The filtrate of one replicate (from a set of triplicates) was collected for carbohydrate analysis to estimate sugar loss during pretreatment. The wet biomass sample from the Buchner funnel was completely transferred to a preweighed zip-locked plastic bag, weighed, and stored sealed. Subsets of the wet pretreated biomass were dried at 105 °C to estimate solid recovery and at 40 °C to determine lignin and carbohydrate contents. Pretreated biomass samples resulting in least lignin content and greatest hemicellulose solubilization relative to untreated samples were hydrolyzed and fermented for ethanol production.

Enzymatic Hydrolysis. The three enzymes, cellulase (NS 50013 cellulase complex, density 1.20 g/mL), cellobiase (NS 50010 β-glucosidase, density 1.24 g/mL), and xylanase (NS50030 xylanase, density 1.09 g/mL), used in this study were kindly provided by Novozymes North America Inc., Franklinton, NC. Cellulase and cellobiase activities measured by enzyme assays[14] were 75.5 FPU (filter paper units)/mL and 634.2 CBU (cellobiase units)/mL, respectively.

Hydrolysis was performed in 50 mL polypropylene centrifuge tubes at 55 °C and 150 rpm for 72 h. Samples were prepared at 5% solid loading by mixing unpretreated or wet pretreated biomass equivalent to 1 g dry basis (determined by moisture content) with 20 mL of 0.05 M citrate buffer (pH 4.8) containing 40 μg/mL tetracycline hydrochloride to avoid microbial contamination. Cellulase activities of 0, 15, and 30 FPU/g dry unpretreated or pretreated biomass were investigated with samples at 0 FPU/g dry biomass used to study the impact of soaking on polysaccharide solubilization. Cellobiase at an activity ratio of 4 CBU/1 FPU (cellobiase/cellulase) was supplemented to avoid end-product inhibition due to cellobiose accumulation.[15,16] The effect of xylanase on reducing sugar generation was studied by adding xylanase at a loading of 0.25% w/w dry biomass to the cellulase and cellobiase combination. Hydrolyzates were centrifuged (Model 5810R, Eppendorf) at 4 °C and 4000 rpm for 10 min, and 1 mL of the supernatant was drawn for fermentable sugar analysis. The remaining hydrolyzates were autoclaved at 121 °C/15 psi for 15 min to inactivate any contaminating microorganisms and degrade the antibiotic before fermentation.

Fermentation. *Saccharomyces cerevisiae* (ATCC 24859), obtained from the Microbiological Engineering Laboratory in the Agricultural and Biological Engineering Department at Pennsylvania State University, was grown in 20 g glucose, 8.5 g yeast extract, 1.32 g NH_4Cl, 0.11 g $MgSO_4$, and 0.06 g $CaCl_2$ per liter of deionized water.[17] The inoculum, containing on average 6.3×10^7 cfu/mL, corresponding to 11 g dry cell weight/L, was prepared by centrifuging 100 mL of actively growing yeast culture at 4000 rpm/4 °C for 10 min, washing the cell pellet twice with 50 mL of 0.1% sterilized peptone water to remove residual media, and resuspending

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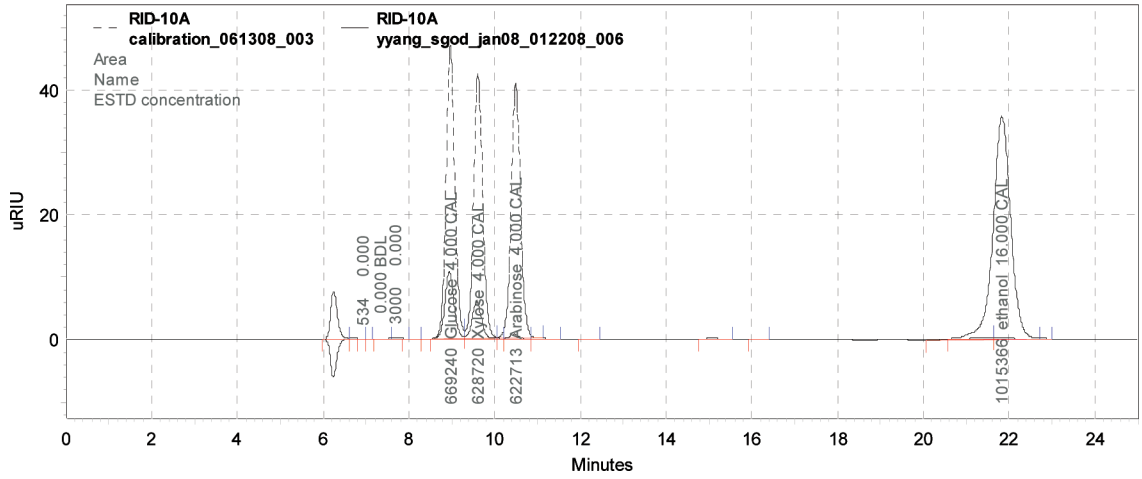

Figure 1. Sample chromatogram of sugar and ethanol analysis using a HPLC. The smaller (solid) peaks represent sugars in a pretreated sample and the larger (dotted) peaks represent a calibration standard.

it in 20 mL of peptone water. Two hundred and fifty microliters of yeast inoculum were added to each tube containing 19 mL of sterilized hydrolyzate for fermentation at 30 °C for 48 h. Fermented samples were centrifuged at 4000 rpm/4 °C for 10 min, and the supernatant was analyzed for ethanol and residual sugars.

Composition Analyses. Composition analyses of unpretreated and pretreated switchgrass germplasms were performed in triplicate. Total solids, ash, acid insoluble lignin (AIL), and acid soluble lignin (ASL) were measured in unpretreated switchgrass according to the National Renewable Energy Laboratory's (NREL) Laboratory Analytical Procedures (LAPs).[18−20] Extractives were determined based on the method described by Han and Rowell.[21] LAP for carbohydrate analysis[20] was modified to determine monomeric sugars (glucose, xylose, and arabinose) using a Shimadzu (Kyoto, Japan) high-performance liquid chromatograph (HPLC) equipped with a refractive index detector (Shimadzu RID-10A). A Biorad Aminex HPX-87H column maintained at 65 °C with a corresponding guard column was used with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min as the mobile phase. Pretreated biomass was characterized for lignin (ASL and AIL) and carbohydrate (based on glucose, xylose, and arabinose) contents, while reducing sugars were measured in hydrolysis and fermentation samples. Ethanol content in the fermentation broth was also measured using the same HPLC procedure but by including ethanol as a standard during calibration. A sample chromatograph showing the sugar and ethanol peaks is presented in Figure 1.

Percent hemicellulose solublization resulting from acid pretreatment was calculated by dividing the difference in xylose plus arabinose contents of unpretreated and pretreated samples by the contents in unpretreated samples alone.

Hydrolysis efficiency was calculated by comparing the glucose yield (g) after hydrolysis with the glucose content (g, or presented as 1.1 times the initial glucan content) in the prehydrolysis biomass (unpretreated or pretreated), using eq 1:[22]

$$\text{Glucan conversion efficiency} = \frac{CVa\dfrac{1\text{ L}}{1000\text{ ml}}}{m \times \%\text{glucan} \times 1.1} \times 100\% \quad (1)$$

Where C is the concentration of glucose after enzymatic hydrolysis detected by HPLC (g/L); V is the volume of the hydrolyzate (ml); a is the dilution rate of hydrolyzate before HPLC sugar analysis; m is the weight of dry biomass (unpretreated or pretreated) before hydrolysis (g); and "% glucan" is based on carbohydrate analysis of the unpretreated or pretreated biomass.

Ethanol yield was calculated by dividing the total amount of ethanol detected (g ethanol) in the fermentation broth by the initial weight of switchgrass (g raw biomass). It was also expressed as percent ethanol yield compared with the theoretical value using eq 2:

$$\text{Percent ethanol yield} = \frac{\text{ethanol(g)/biomass(g)}}{0.511 \times \text{glucose(g)/biomass(g)}} \quad (2)$$

Where 0.511 is the theoretical ethanol yield (g) generated per gram of glucose;[23] ethanol (g)/biomass (g) is the the ethanol yield (g) per 1 g of unpretreated biomass; and glucose (g)/biomass (g) is the glucose content (g) in 1 g of unpretreated biomass.

Statistical Analysis. Multiple comparisons with the best[24] were conducted using SAS (version 9.1.3, Cary, NC) to identify the most effective pretreatment condition for each germplasm based on "least lignin content" and "greatest hemicellulose solubilization". Triplicate pretreatment data (three acid concentrations × three residence times) for each of the three germplasms was used for analysis. Analysis of variance (ANOVA) was performed to determine effects of factors such as acid concentration and treatment time on pretreatment and cellulase activity levels and xylanase addition on hydrolysis and fermentation. A 95% confidence level was used for all analyses performed in this study. Additionally, Tukey simultaneous tests were conducted to test the statistical differences between treatments.

Results and Discussion

Characteristics of Switchgrass. Oven-dried switchgrass samples of three different germplasms were analyzed for key components (Table 1) and were found to contain 26.65−29.28% glucan, 17.92−19.37% xylan, 2.02−2.76% arabinan, and 17.74−19.23% lignin on dry matter basis. Moisture content of the oven-dried samples ranged from 1.24 to 1.54%. Acid

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Table 1. Composition of Three Oven-dried Switchgrass Germplasms[a]

composition	switchgrass germplasm		
	St6−1	St6−3E	St6−3F
ash	3.07 (0.06)	2.98 (0.01)	3.88 (0.05)
extractives	6.40 (0.32)	5.38 (0.25)	4.87 (0.41)
acid insoluble lignin	15.93 (0.06)	15.50 (0.22)	13.70 (0.27)
acid soluble lignin	3.30 (0.09)	3.48 (0.15)	4.04 (0.16)
glucan	26.65 (4.44)	27.24 (0.35)	29.28 (0.88)
xylan	18.06 (2.66)	17.92 (0.35)	19.37 (0.81)
arabinan	2.10 (0.42)	2.02 (0.17)	2.76 (0.03)
others	24.49	25.48	22.10

[a] Averages expressed on % dry matter basis with standard deviation in parentheses.

insoluble lignin contributed approximately 80% of the total lignin content of switchgrass. For the three germplasms studied, glucan and xylan contributed approximately 50% of the total dry matter, while arabinan was measured at 2−3% of the total dry matter of switchgrass. Compared with commercial switchgrass varieties like Alamo and Cave-in-Rock, both ash and extractives were less in the three germplasms studied.[25]

The remaining undefined components in the samples tested could possibly be organic compounds such as uronic acid, acetyl groups, and several other minerals[26−28] and residual extractives such as waxes, fats, gums, starches, resins, and essential oils.[29] Overall, compared with St6−3F, the compositions of St6−1 and St6−3E were more comparable.

Effect of Dilute Acid Pretreatment. Switchgrass germplasms pretreated with dilute sulfuric acid at 0.5, 1.0, and 1.5% (w/v) for 30, 45, and 60 min were analyzed for sugar and lignin contents and the results are summarized in Tables 2−4. Sulfuric acid pretreatment resulted in 51.97−72.56% solid recovery for the three switchgrass germplasms. Solid recovery was significantly affected by germplasm, treatment time, and acid concentration ($P < 0.05$) with recovery decreasing as the acid concentration and residence time increased.

Effect of Pretreatment on Lignin Degradation. Sulfuric acid pretreatment did not greatly remove lignin from switchgrass even under intense pretreatment conditions (concentration and time). Statistically, for each germplasm, acid concentration, residence time, and their interaction (acid × time) did not significantly impact ($P > 0.05$) lignin content in the pretreated samples except the interaction term for St6−1 ($P < 0.05$). For St6−1, St6−3E, and St6−3F switchgrass samples, the greatest lignin removal was limited to 5.46, 8.12, and 6.54%, respectively (Tables 2−4). Lignin has been reported to be very sensitive to sulfuric acid and two opposite mechanisms, degradation (depolymerization) and accumulation (repolymerization), can occur simultaneously when lignin interacts with sulfuric acid.[30,31] Yang and Wyman[32] reported that lignin removal during dilute acid pretreatment in a batch process was less than that with hot water treatment alone at the same treatment temperature. In this study lignin accumulation was possibly greater than lignin degradation during acid pretreatment of the selected germplasms. Since the switchgrass samples were not extracted prior to pretreatment, it is also probable that those chemicals (mostly the organic compounds) condensed with lignin during H_2SO_4 treatment and were measured as acid insoluble lignin in the postpretreatment analysis.[33,34] Presence of any component, besides acid soluble lignin, in the liquid fraction that can impact the absorption of light at 205 nm may have also lead to an overestimation of the lignin content.[35]

Effect of Pretreatment on Hemicellulose Solubilization. Unlike lignin, the amount of hemicellulose in pretreated biomass samples decreased as intensity of pretreatments (higher acid concentration and/or longer residence time) increased during dilute acid pretreatment. ANOVA demonstrated that acid concentration, significantly ($P < 0.05$) influenced solubilization of hemicellulose in all three germplasms. However, residence time and the interaction term (acid × time) were not significant for any of the germplasms ($P > 0.05$).

The degree of hemicellulose solubilization in pretreated solids increased significantly from 32.96−36.45% when treated with the mildest condition (30 min and 0.5% H_2SO_4) to 76.32−83.58% when subjected to the most severe pretreatment (60 min and 1.5% H_2SO_4) (Tables 2−4). Other studies on dilute acid pretreatment have shown that hemicellulose in lignocelluloses can be completely solubilized during acid pretreatment under severe reaction conditions.[5,36−38] A greater level of hemicellulose solubilization can enhance the digestibility of cellulose in residual solids, thus increasing the enzymatic conversion efficiency.[4]

Analysis of reducing sugars in the filtrate from pretreated switchgrass samples showed that up to 127.88−147.57 mg/g xylose was present in the filtrate of the three germplasms.[39] The filtrate from a non-acid (deionized water) pretreatment control performed on St6−3F at 10% solid loading and autoclaved for 30 min contained only 5.72 mg xylose out of 193.7 mg xylan per gram of raw St6−3F switchgrass. This suggests that, without acid, moderately high temperature and pressure are ineffective in solubilizing hemicellulose.

Apart from the three monomeric sugar peaks, other unidentified component peaks were also eluted during HPLC analysis of the filtrate. These peaks may be representative of smaller components such as furfural derived from the degradation of monomeric sugars during acid pretreatment,[4,6,40] but they were not monitored in this study.

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Table 2. Composition of St6-1 Oven-dried Switchgrass after Acid Pretreatment at Different Conditions[a]

pretreatment conditions		composition of solid fractions[b] (%)					
time (min)	acid concentration (%)	total solid[b]	lignin	glucose	xylose	arabinose	hemicellulose[b] solubilization (%)
unpretreated sample		100	19.22 (0.11)	29.31 (4.88)	19.87 (2.93)	2.31 (0.46)	
30	0.5	72.56 (1.71)	19.14 (0.38)	25.54 (3.77)	13.68 (3.50)	0.79 (0.16)	34.74 (16.51)
	1.0	60.92 (0.97)	18.38 (0.55)	22.39 (6.78)	7.59 (3.15)	0.30 (0.15)	64.40 (14.90)
	1.5	57.75 (1.20)	18.53 (0.48)	25.37 (8.22)	5.90 (1.37)	0.31 (0.12)	71.99 (6.48)
45	0.5	68.74 (1.19)	18.80 (0.32)	20.95 (6.21)	9.65 (3.10)	0.38 (0.13)	54.77 (14.56)
	1.0	58.93 (1.63)	18.50 (0.38)	28.88 (7.93)	6.99 (1.57)	0.38 (0.18)	66.76 (7.92)
	1.5	56.73 (0.85)	19.05 (0.46)	28.73 (11.00)	5.92 (2.06)	0.28 (0.14)	72.05 (9.91)
60	0.5	63.71 (0.98)	18.17 (0.72)[c]	22.74 (9.33)	8.84 (4.54)	0.42 (0.30)	58.26 (21.81)
	1.0	56.22 (0.11)	18.48 (0.57)	21.57 (3.60)	4.80 (0.86)	0.24(0.11)	77.24 (4.25)[d]
	1.5	54.60 (0.55)	19.03 (0.42)	28.77 (6.00)	4.95 (0.71)	0.30 (0.09)	76.32 (3.62)

[a] Expressed as g/100g initial dry switchgrass. [b] Expressed as Mean value (standard deviation). [c] Least lignin availability observed. [d] Greatest hemicellulose solubilization observed.

Table 3. Composition of St6-3E Oven-dried Switchgrass after Acid Pretreatment at Different Conditions[a]

pretreatment conditions		composition of solid fractions[b] (%)					
time (min)	acid concentration (%)	total solid[1]	lignin	glucose	xylose	arabinose	hemicellulose[1] solubilization (%)
unpretreated sample		100	18.97 (0.38)	29.96 (0.38)	19.71 (0.39)	2.22 (0.19)	
30	0.5	70.65 (2.44)	17.94 (0.44)	24.05 (0.72)	13.26 (0.70)	0.69 (0.03)	36.45 (3.34)
	1.0	60.29 (1.96)	17.43 (0.46)[c]	23.36 (7.76)	7.25 (3.98)	0.33 (0.24)	65.43 (19.20)
	1.5	56.61 (0.59)	17.74 (0.68)	32.18 (4.64)	7.26 (0.09)	0.37 (0.01)	65.24 (0.43)
45	0.5	64.60 (5.50)	17.56 (0.05)	31.28 (7.42)	13.66 (3.43)	0.66 (0.32)	34.77 (17.08)
	1.0	58.48 (0.99)	17.60 (0.34)	25.62 (4.26)	6.68 (1.23)	0.28 (0.13)	68.26 (6.22)
	1.5	55.48 (1.02)	18.09 (0.19)	27.28 (4.04)	5.35 (1.23)	0.28 (0.13)	74.35 (6.20)
60	0.5	63.22 (0.56)	18.30 (0.02)	28.60 (8.70)	10.22 (3.17)	0.55 (0.24)	50.94 (15.53)
	1.0	55.47 (0.49)	17.51 (0.94)	28.86 (0.97)	6.09 (0.12)	0.36 (0.01)	70.58 (0.58)
	1.5	53.63 (0.37)	18.07 (0.43)	20.27 (5.59)	3.43 (1.11)	0.17(0.00)	83.58 (5.07)[d]

[a] Expressed as g/100g initial dry switchgrass. [b] Expressed as Mean value (standard deviation). [c] Least lignin availability observed. [d] Greatest hemicellulose solubilization observed.

Table 4. Composition of St6-3F Oven-dried Switchgrass after Acid Pretreatment at Different Conditions[a]

pretreatment conditions		composition of solid fractions[b](%)					
time (min)	acid concentration (%)	total solid[b]	lignin	glucose	xylose	arabinose	hemicellulose[b] solubilization (%)
unpretreated sample		100	17.74 (0.25)	32.21 (0.97)	21.31 (0.89)	3.04 (0.03)	
30	0.5	71.91 (2.31)	17.55 (0.24)	27.76 (2.82)	15.39 (0.19)	0.94 (0.04)	32.96 (0.94)
	1.0	58.57 (1.42)	16.58 (0.50)[c]	28.28 (8.49)	8.72 (2.37)	0.44 (0.11)	62.36 (10.12)
	1.5	54.78 (1.46)	16.86 (0.21)	23.15 (10.01)	5.46 (3.22)	0.32 (0.21)	76.21 (14.07)
45	0.5	65.98 (2.77)	16.85 (0.55)	29.68 (8.26)	12.41 (3.59)	0.62 (0.27)	46.46 (15.87)
	1.0	55.65 (2.06)	17.41 (0.34)	31.39 (7.17)	7.87 (1.81)	0.46 (0.14)	65.79 (8.03)
	1.5	54.41 (0.73)	16.80 (0.00)	22.28 (5.47)	4.34(1.60)	0.24 (0.10)	81.20 (6.92)[d]
60	0.5	62.10 (1.09)	17.18 (0.18)	27.60 (7.41)	10.76 (3.13)	0.54 (0.24)	53.59 (13.82)
	1.0	54.62 (1.15)	17.10 (0.74)	27.10 (4.78)	5.79 (0.77)	0.30 (0.10)	75.02 (3.55)
	1.5	51.97 (0.34)	16.71 (0.37)	27.71 (6.73)	4.72 (0.93)	0.23 (0.10)	79.69 (4.24)

[a] Expressed as g/100g initial dry switchgrass. [b] Expressed as Mean value (standard deviation). [c] Least lignin availability observed. [d] Greatest hemicellulose solubilization observed.

Effect of Pretreatment on Other Cell Wall Compounds. Statistical analysis indicated that acid concentration, residence time, and their interaction term did not have significant effects ($P > 0.05$) on the cellulose (glucose) content in pretreated switchgrass for all three germplasms. The majority of the cellulose was retained in the solid residue and ranged from 20.27−32.21% on raw dry matter basis depending on pretreatment condition and germplasm (Tables 2−4). The percentage of cellulose released to the liquid fraction during acid pretreatment was limited to 28.52, 32.34, and 30.83% (obtained by dividing the maximum difference in glucose content of pretreated and unpretreated biomass by the glucose content in unpretreated biomass) for St6−1, St6−3E, and St6−3F switchgrass samples, respectively.

Apart from lignin, hemicellulose, and cellulose, plant cells contain proteins and extractives such as waxes. It has been reported that extractives can be removed simultaneously with hemicellulose during acid pretreatment.[41] Also, the protein content decreases as acid concentration, reaction temperature, and time increase during dilute sulfuric acid pretreatment.[36] In this study, extractives and proteins remaining in the pretreated solids were not quantified. However, as evident from the post-pretreatment composition analysis (Tables 2−4), the sum of lignin and all three sugars approximately equaled the total solids (after pretreatment) as treatment conditions became more intense, thus indicating that proteins and extractives may have been removed from the solid biomass under such severe conditions.

Enzymatic Hydrolysis. Switchgrass samples with the least percent of lignin content or greatest percent hemicellulose solubilization for each germplasm were hydrolyzed with cellulase, cellobiase, and xylanase at various activity levels.

Hydrolysis of the Pretreated Least Lignin Content Biomass. Three pretreatments that provided least lignin content—60 min and 0.5% acid for St6−1, and 30 min and 1.0% acid for both St6−3E and St6−3F—were selected for enzymatic hydrolysis and fermentation on the basis of multiple comparisons with the best (SAS 9.1.3, Cary, NC). Glucan conversion efficiency in least lignin content samples, summarized in Figure

(41) Nguyen, Q. A.; Tucker, M. P.; Keller, F. A.; Eddy, F. P. *Appl. Biochem. Biotechnol.* **2000**, *84−86*, 561–576.

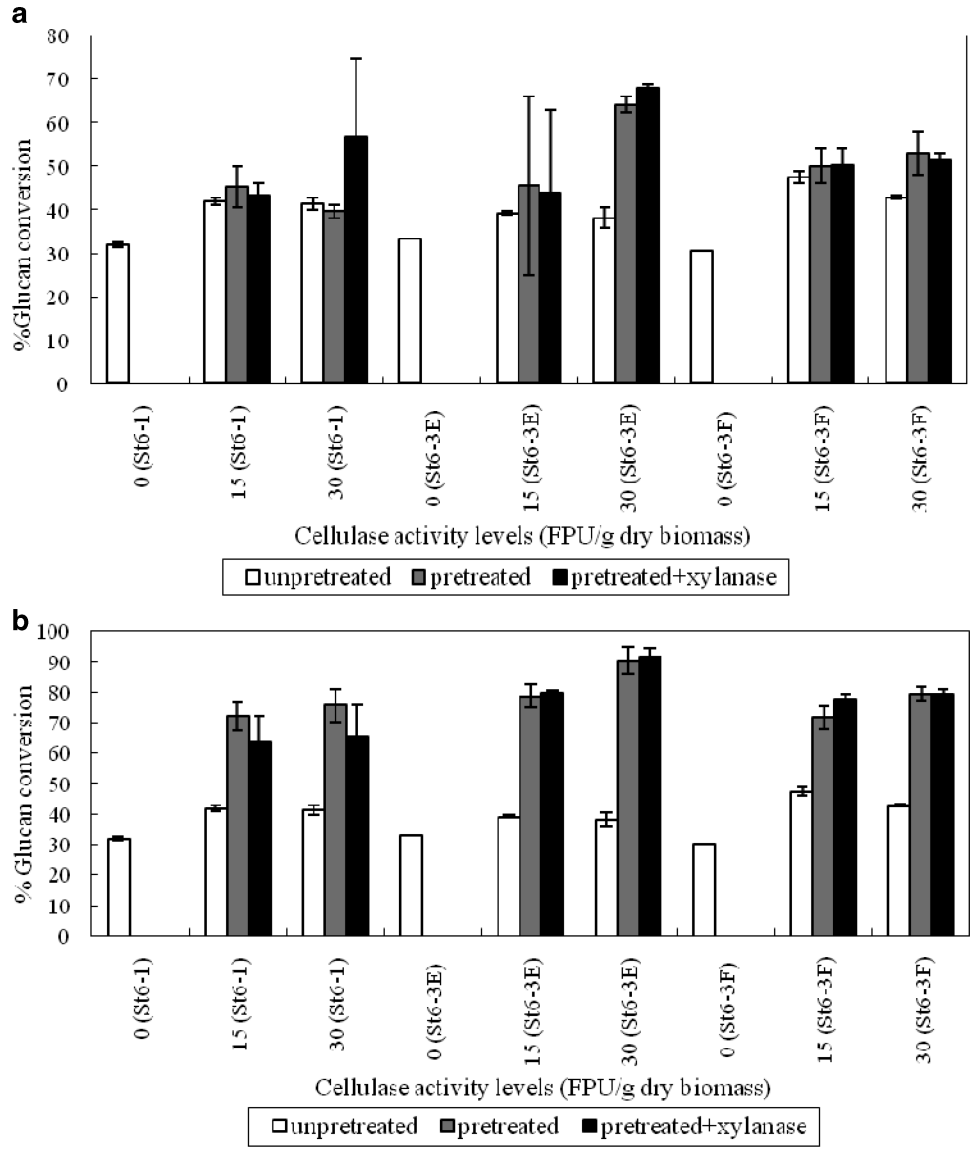

Figure 2. Hydrolysis of oven-dried switchgrass germplasms with (a) least lignin content and (b) greatest hemicellulose solubilization. Data for unpretreated samples are the same as those in Figure 2a and b.

2a, shows the effect of pretreatment, cellulase/cellobiase addition, and loading as well as xylanase addition on reducing sugar yield.

The enzymatic conversion of pretreated glucan was between 39.60 and 68.02% compared with 30.55−47.56% for unpretreated biomass. Adding cellulase significantly increased hydrolysis efficiency compared with the noncellulase control for all three germplasms. However, no statistical differences in percent glucan conversion were observed between the cellulase loadings of 15 FPU and 30 FPU/g dry biomass ($P > 0.05$) except for St6−3E samples. Pretreatment did not have a significant impact on hydrolysis efficiency ($P > 0.05$) when the no-enzyme control data was included for statistical analysis. Öhgren et al.[42] showed that supplementation of the cellulase mixture with xylanase at a concentration of 0.06 g protein/g cellulose increased hemicellulose hydrolysis with the xylose yield increasing from 65 to 90% of theoretical, thus making cellulose more accessible to cellulase. However, in this study, adding xylanase did not significantly improve cellulose conversion ($P > 0.05$), especially at a cellulase loading of 15 FPU/g dry biomass. An exception was St6−1, in which, at a cellulase loading of 30 FPU/g dry biomass, xylanase significantly promoted ($P < 0.05$) glucan-to-glucose conversion.

Generally, no cellobiose was detected in the hydrolyzate, indicating effective conversion by cellobiase, thus preventing end-product inhibition. In the hydrolyzate from control samples without enzymes, pretreated switchgrass did not generate any monomeric sugars (glucose, xylose, and arabinose). However, 30.55−33.24% glucan conversion was detected in the unpretreated control without enzymes. This may have been due to the transfer of all free monomeric sugars from pretreated solids into the pretreatment liquid or deionized water used for washing samples, thus leaving no free sugars on the surface of pretreated biomass.

Hydrolysis of the Pretreated Greatest Hemicellulose Solubilization Biomass. For each germplasm, the pretreatment condition that led to the greatest percent hemicellulose (both xylose and arabinose) solubilization based on a multiple comparison analysis (SAS 9.1.3, Cary, NC) was selected for subsequent hydrolysis. The treatment conditions were 60 min and 1.0% acid for St6−1; 60 min and 1.5% acid for St6−3E; and 45 min and 1.5% acid for St6−3F. Compared to unpre-

(42) Öhgren, K.; Bura, R.; Saddler, J.; Zacchi, G. *Biores. Technol.* **2007**, *98*, 2503–2510.

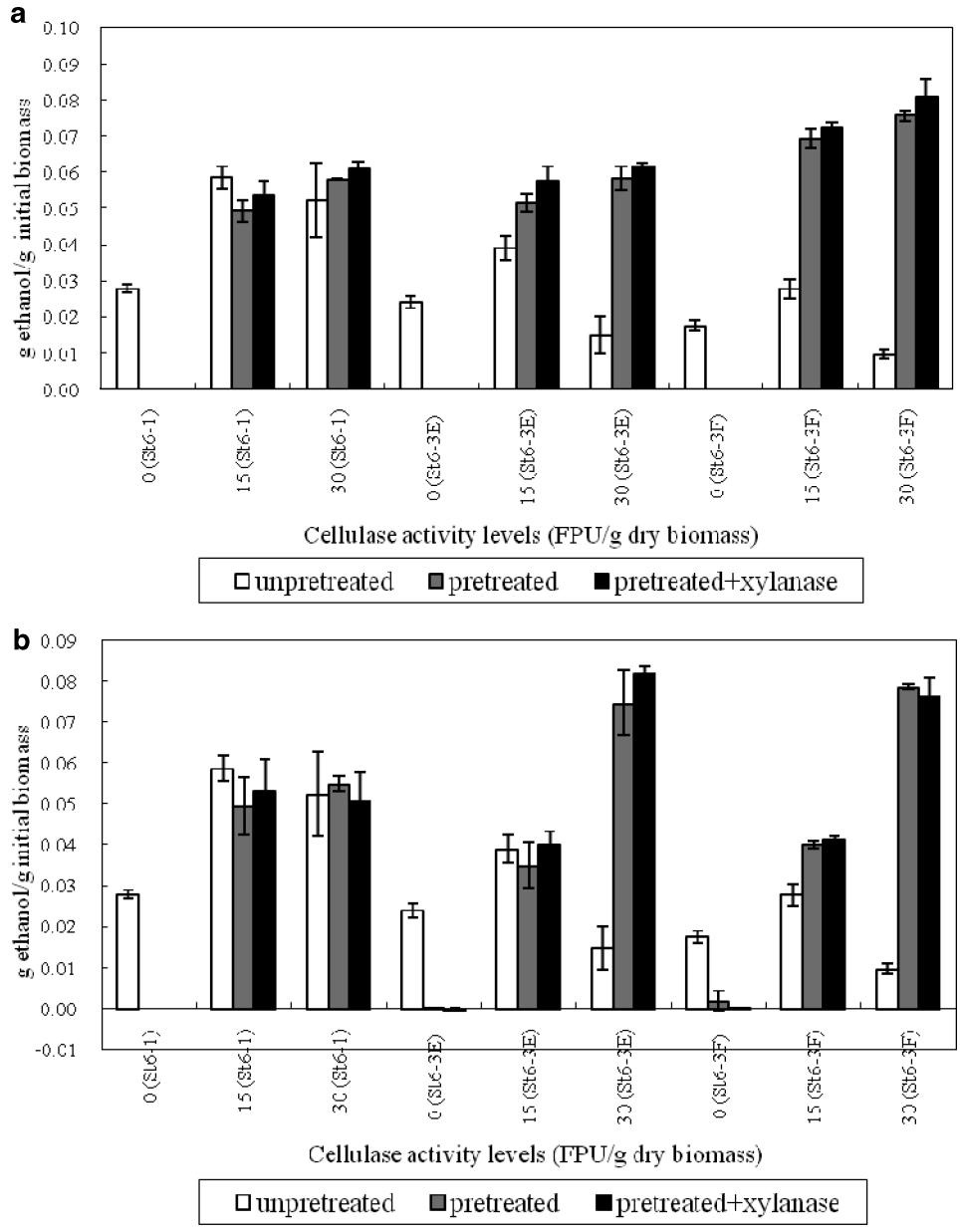

Figure 3. Ethanol yields from oven-dried switchgrass germplasms with (a) least lignin content after pretreatment and (b) greatest hemicellulose solubilization after pretreatment. Data for unpretreated samples are the same as those in Figure 3a and b.

treated samples that showed a maximum glucan conversion of 47.56%, pretreated samples had a conversion efficiency greater than 64%, with the greatest being 91.75% for St6−3E pretreated samples hydrolyzed with cellulase at 30 FPU/g dry biomass plus xylanase (Figure 2b). No monomeric sugars were detected in the liquid fraction of the no-enzyme control. Pretreatment enhanced glucan conversion significantly ($P < 0.05$) for all three germplasms when no-enzyme control data was not included in the analysis. However the increase was significant ($P < 0.05$) only for St6−3E when control data was included, probably due to the overriding effect of samples with no monomeric sugars in the hydrolysate. It was observed that, compared with delignification, hemicellulose removal is a more effective approach for improving glucan conversion efficiency during enzymatic hydrolysis of switcghrass.

Xylan Conversion during Enzymatic Hydrolysis. Xylan conversion efficiency during hydrolysis of unpretreated biomass ranged from 31.31 to 41.22% (data not shown).[39] It was interesting to note that xylan conversion in pretreated samples was not significantly different ($P > 0.05$) from that in unpretreated samples, except pretreated least-lignin-content St6−1 and St6−3F germplasms, which showed statistically lower xylan-to-xylose conversion. Addition of xylanase did not significantly enhance xylan hydrolysis ($P > 0.05$), whereas the addition of cellulase statistically affected xylan conversion ($P < 0.05$). This could be because the cellulase enzyme complex contains sufficient xylanase activity for residual xylan since the majority of xylan was removed during acid pretreatment and any subsequent xylanase addition is not necessary.[37] Alizadeh et al.[43] and Chen et al.[17] observed that high xylan conversion efficiency during hydrolysis could be achieved by cellulase digestion without xylanase.

Fermentation. Hydrolyzates were fermented with a non-xylose-metabolizing yeast strain to estimate the potential bioethanol yield from switchgrass based on cellulose (glucose) conversion (Figure 3, panels a and b).

Fermentation of Hydrolyzates from Pretreated Least Lignin Content Biomass. Ethanol yields ranging from 0.049 to 0.081 g/g raw biomass were obtained from hydrolyzates of pretreated

switchgrass samples with least lignin content. No ethanol was detected in hydrolyzates of pretreated samples hydrolyzed in the absence of enzymes (Figure 3a). Pretreatment had a significant effect on ethanol yield from St6−3F ($P < 0.05$). Pretreated St6−3E samples, when hydrolyzed with cellulolytic enzymes, showed improved ethanol yields compared to unpretreated samples; however, the effect of pretreatment was not significant ($P > 0.05$) when the no-enzyme controls were included in analysis. Acid pretreated St6−3F switchgrass produced approximately 2−5 times more ethanol than the unpretreated samples, indicating that effective improvement in ethanol yield for this specific germplasm can be achieved by increasing the digestibility of the biomass through dilute acid pretreatment. The addition of cellulase enhanced final ethanol yield ($P < 0.05$) compared to no-enzyme controls. Although increasing cellulase loading from 15 to 30 FPU/g dry biomass and addition of xylanase did not significantly improve ethanol production ($P > 0.05$), within each germplasm, the greatest ethanol yield was obtained with pretreated biomass hydrolyzed at a cellulase loading of 30 FPU/g dry biomass supplemented with xylanase.

Glucose released during enzymatic hydrolysis of all pretreated samples was almost completely consumed by yeast within 48 h of fermentation, but there was no obvious change in xylose content during fermentation. Therefore, although the yeast being used in this study (*Saccharomyces cerevisiae*, ATCC 24859) was not capable of simultaneously converting xylose to ethanol, it was robust enough to efficiently consume glucose to produce ethanol without inhibition. Additionally, in this study, all pretreated switchgrass samples displayed high fermentation efficiency possibly due to the effective washing of biomass after acid pretreatment for removing potential fermentation inhibitors such as furfurals that may have been generated during pretreatment.

Fermentation of Hydrolyzates from the Pretreated Greatest Hemicellulose Solubilization Biomass. Ethanol yields ranging from 0.035 to 0.082 g/g raw biomass were obtained from hydrolyzates of pretreated switchgrass samples with high hemicellulose solubilized. No ethanol was detected in hydrolyzates of no-enzymes controls except pretreated St6−3F in which 0.45 g/L ethanol was detected (Figure 3b).

Pretreatment enhanced the biomass-to-bioethanol conversion for St6−3E and St6−3F switchgrass. The improvement was statistically significant for St6−3F ($P < 0.05$) as the ethanol yield from the hydrolyzate obtained with cellulase at 30 FPU/g dry biomass reached 0.079 g/g raw biomass compared with only 0.010 g of ethanol obtained from unpretreated biomass hydrolyzed at similar cellulase loadings. Addition of cellulase significantly influenced ethanol yield for all three germplasms ($P < 0.05$). Furthermore, increasing cellulase activity from 15 to 30 FPU/g dry biomass during hydrolysis significantly promoted ethanol production for the St6−3F germplasm ($P < 0.05$). Adding xylanase during hydrolysis, however, did not impact the final ethanol yield statistically ($P > 0.05$). Apart from the presence of adequate xylanase activity in the cellulase complex itself, this effect may be attributed to the inability of conventional yeast to ferment xylose due to which generation of any additional xylose during hydrolysis does not necessarily improve final ethanol yield.

Ethanol produced per gram of non-pretreated St6−1 switchgrass through direct hydrolysis and fermentation ranged from 0.052 to 0.059 g, and the yield was similar to that obtained from pretreated St6−1 switchgrass sample. Comparable ethanol yields (0.070−0.080 g ethanol/g initial biomass) were obtained from pretreated St6−3F samples with the least lignin content and greatest hemicellulose solubilization hydrolyzed by cellulase at 30 FPU/g dry biomass. However, the most severe pretreatment condition (60 min and 1.5% H_2SO_4), under which the greatest hemicellulose solubilization was achieved for St6−3E switchgrass, resulted in a much greater ethanol yield (0.075−0.082 g ethanol/g initial biomass) compared with the least lignin content sample (0.052−0.062 g ethanol/g initial biomass) within this germplasm.

Evaluation of Ethanol Production Potential of Switchgrass. The optimal ethanol yield obtained from acid-pretreated St6−1, St6−3E, and St6−3F germplasms was 40.7, 53.5, and 49.0% of the theoretical yield, respectively. These values are less than 72% of the theoretical ethanol yield reported from SSF of lime-pretreated switchgrass.[44] The highest ethanol yield was 0.082 g ethanol/g raw biomass (considering glucose fermentation only) for St6−3E switchgrass pretreated with 1.5% (w/v) sulfuric acid for 60 min and hydrolyzed by cellulase at 30 FPU/g dry biomass supplemented with xylanase. It was less than the optimized yield (0.2 g ethanol/g dry biomass) reported by Alizadeh et al.[42] in which the switchgrass was first pretreated by ammonia fiber explosion (AFEX) and then simultaneously saccharified and fermented (SSF) with a nonpentose-fermenting yeast strain. Besides the difference that this study evaluated separate hydrolysis and fermentation, lower ethanol yield could be due to loss of some cellulose during acid pretreatment compared with lime pretreatment.

Conclusion

Three new switchgrass germplasms containing 26.65−29.28% glucan, 17.92−19.37% xylan, and 17.74−19.23% lignin were investigated for their ethanol production potential. The acid pretreatments yielding greatest hemicellulose solubilization generally involved severe conditions such as longer residence time (60 min) or greater acid concentration (1.5% H_2SO_4), whereas those providing the least lignin contents were less intense with the effect of acid pretreatment on delignification not being apparent. Hydrolysis and fermentation of the pretreated biomass indicated that the selected pretreatments were effective at enhancing subsequent enzymatic hydrolysis and final ethanol yield. Glucose produced during enzymatic hydrolysis was almost completely consumed during fermentation, and the greatest ethanol yield of 0.082 g ethanol/g raw St6−3E or St6−3F averaged approximately 50% of theoretical value. Overall, among the three new switchgrass germplasms studied, St6−3E and St6−3F displayed good potential for bioethanol production. These results demonstrate that the new switchgrass cultivars being studied can be potential energy crops for producing bioethanol. However, investigation of other pretreatment methods, scale-up, and economic analysis is required to draw further conclusions.

Acknowledgment. The authors would like to thank Dr. Jason A. Osborne (Department of Statistics, NCSU) for assisting with the statistical analysis. Also, valuable suggestions from Dr. George C. Allen (Department of Horticultural Science, NCSU) are greatly appreciated. Funding for this project was provided by the North Carolina Agricultural Research Service (NCARS).

EF900043Z

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