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Saccharification and Fermentation of Dilute-Acid-Pretreated Freeze-Dried Switchgrass†

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This study investigated the potential of three freeze-dried switchgrass germplasms (St6-1, St6-3E, and St6-3F) as whole plants or their stems and leaves for bioethanol production. Whole switchgrass germplasms contained 24.34−30.95% glucan, 14.68−18.58% xylan, and 17.39−19.46% lignin. Switchgrass samples were pretreated with dilute sulfuric acid at concentrations of 0.5, 1.0, or 1.5% (w/v) for 30, 45, or 60 min at 121 °C and 15 psi. Although lignin degradation was limited, over 80% hemicellulose solublization was observed, especially in leaf samples, and the removal could be enhanced by increasing the pretreatment intensity through acid concentration and treatment time adjustment. Within each germplasm, pretreated samples with the least lignin content or greatest percent hemicellulose (xylan and arabinan) solublization were hydrolyzed enzymatically by cellulase at 0, 15, or 30 filter paper units (FPU)/g of dry biomass supplemented with cellobiase. Although the addition of cellulase greatly improved cellulose to glucose conversion, no significant ($p \geq 0.05$) differences were observed between activity levels of 15 and 30 FPU/g of dry biomass. Pretreatment significantly ($p < 0.05$) improved cellulose conversion in samples with the greatest hemicellulose solublization; complete cellulose hydrolysis was observed in some St6-3F samples. Fermentation of hydrolyzates with *Saccharomyces cerevisiae* (ATCC 24859) resulted in the greatest ethanol yield of 0.083 g of ethanol/g of raw St6-3F switchgrass whole plant, which was 60% of the theoretical yield. Results from this study demonstrated the potential of new switchgrass germplasms as energy crops for bioethanol production through dilute sulfuric acid pretreatment.

Introduction

Rapid growth in the global energy demand and serious concerns over greenhouse gas emissions focus on an urgent need to explore renewable energy sources and improve the efficiency of energy generation processes. Because liquid fuels are vital for transportation, a key goal of sustainable energy development is to produce alternative environmentally friendly fuels capable of replacing petroleum-based liquids. Among various renewable energy sources, such as biomass, solar, wind, and hydropower, biomass-derived fuels have emerged as competitive candidates, meeting over 3% of the United States energy needs, thus surpassing hydropower to become the number 1 renewable energy resource in the U.S.[1]

Biomass-based fuels include biodiesel and bioethanol. Currently, the commercial production of bioethanol relies largely on conversion of starch from corn kernels in the U.S. and sucrose from sugar cane in Brazil. However, these two feedstocks tap into food supply chains and are limited by fertile land occupation as well as rigid requirements for climate.[2,3] An alternative is lignocellulosic biomass, which is capable of making bioethanol production more cost-effective because of its widespread availability with relatively low input.[4] Lignocellulosic feedstocks can be agricultural or forestry residues as well as bioenergy crops, such as switchgrass.

Switchgrass is a native warm-season, perennial grass and has a wide range of geographic adaptation in North America.[5] It uses the C_4 photosynthesis route, resulting in greater accumulation of dry carbon mass at a lower cost compared to most other C_3 woody and herbaceous species. It is rich in cellulose [approximately 30% on a dry matter basis (db)] and contains less ash and moisture than woods, thus making it suitable for producing bioethanol.[6−8] Conversion of switchgrass to bioethanol comprises (1) pretreatment to disrupt the close interaction between plant cell wall components,

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(1) Perlack, R. D.; Wright, L. L.; Turhollow, A. F.; Graham, R. L.; Strokes, B. J.; Erbach, D. C. Biomass as feedstock for a bioenergy and bioproducts industry: The technical feasibility of a billion-ton annual supply, 2005 (available online at http://feedstockreview.ornl.gov/pdf/billion_ton_vision.pdf).

(2) Parikka, M. Global biomass fuel resources. *Biomass Bioenergy* **2004**, *27*, 613–620.

(3) Gray, K. A.; Zhao, L.; Emptage, M. Bioethanol. *Curr. Opin. Chem. Biol.* **2006**, *10*, 141–146.

(4) von Sivers, M.; Zacchi, G. Ethanol from lignocellulosics: A review of the economy. *Bioresour. Technol.* **1996**, *56*, 131–140.

(5) Phillips, R.; Rix, M. *Perennials*; Firefly Books, Inc.: New York, 2002; Vol. 2, Late Perennials.

(6) McKendry, P. Energy production from biomass (part 1): Overview of biomass. *Bioresour. Technol.* **2002**, *83*, 37–46.

(7) McLaughlin, S.; Bransby, B. D.; Conger, B.; Ocumpaugh, W.; Parrish, D.; Taliaferro, C.; Vogel, K.; Wullschleger, S. Developing switchgrass as a "bioenergy crop". In *Perspectives on New Crops and New Uses*; ASHS Press: Alexandria, VA, 1999; pp 282−299.

(8) van den Oever, M. J. A.; Elbersen, H. W.; Keijsers, E. R. P.; Gosselink, R. J. A.; De Klerk-engels, B. Switchgrass (*Panicum virgatum* L.) as a reinforcing fibre in polypropylene composites. *J. Mater. Sci.* **2003**, *38*, 3697–3707.

including lignin, hemicellulose, and cellulose, (2) hydrolysis to break down polysaccharides into reducing sugars, and (3) fermentation to convert reducing sugars to ethanol. Dilute sulfuric acid pretreatment at elevated temperatures is a method used to convert hemicellulose in lignocellulosic biomass to soluble sugars, thus increasing the efficiency of subsequent enzymatic cellulose digestion.[9,10] Varga et al.[11] reported that hemicellulose reduction in lignocellulosic feedstocks, such as corn stover, could reach 85% upon mixing the biomass with 2% (w/w) sulfuric acid for one night followed by steam treatment at 190 °C for 2 min. Lignin degradation is however not as extensive as with alkaline pretreatments.[12,13] Relative to switchgrass, Torget et al.[14] found that 92% of switchgrass xylan could be solublized upon pretreatment with 0.5% (v/v) sulfuric acid at 140 °C for 60 min or at 160 °C for 10 min.

Although acid pretreatment of lignocellulosic biomass has been widely studied, limited work has been performed to investigate the effect of feedstock processing/drying methods on pretreatment efficiency and the impact of plant parts on bioethanol production, especially for switchgrass. Therefore, the goal of this study was to determine the varying effects of dilute acid pretreatment on the ethanol production from three freeze-dried switchgrass germplasms as impacted by differences in plant part composition. The effect of chemical concentration and reaction time on lignin and hemicellulose solublization as impacted by germplasm and type of plant sample was investigated. The effect of cellulolytic enzyme loadings and combinations on the conversion of cellulose to glucose during enzymatic hydrolysis of select pretreated switchgrass samples was also studied. The final ethanol yield was determined to evaluate the potential of the entire switchgrass-to-ethanol process.

Experimental Section

Switchgrass Feedstock. Three switchgrass germplasms (St6-1, St6-3E, and St6-3F), each adapted to the southeastern U.S. and from well-established stands, were harvested from the Central Crops Research Station, Clayton, NC, in August 2005. Additionally, whole-plant switchgrass for all three germplasms harvested in July 2007, with composition statistically similar ($p \geq 0.05$) to those from 2005, were used in some experiments. Since sampling, St6-3E (selected for high dry matter yield) and St6-3F (improved digestibility) have been released as "BoMaster" and "Performer", respectively.[15,16] Digestibility and dry matter yield are not associated positively;[16] however, at the time of sampling for this study, all three entries provided comparable dry matter yields of approximately 13 450 kg/ha (12 000 lb/acre).

Switchgrass germplasms were harvested randomly to about 6 in. stubble from each field, and weighed small sample portions were taken to determine the moisture content and thus dry matter yield per unit land area. Each bulk sample representing a specific germplasm was divided into two sub-samples. One was retained as the whole-plant bulk, while the other was separated into stems and leaves. Whole, stem, and leaf samples were placed in sealed Ziplock plastic bags and submerged in liquid nitrogen for immediate freezing and cessation of respiration. Samples were removed after 30 min, transferred into a freezer drier at −20 °C, and held for 6 days until they were freeze-dried (model FFD-40-WS, The Virtis Company, Inc., Gardiner, NY). Upon completion of drying, samples were reduced in size by grinding in a Wiley mill fitted with a 2 mm screen. Ground samples, collected in sealed plastic bags, were delivered to the Biological and Agricultural Engineering Department at North Carolina State University, Raleigh, NC, where they were stored at −80 °C until use.

Dilute Acid Pretreatment. Whole plant, stem, and leaf switchgrass samples (3 germplasms × 3 sample types) were pretreated with sulfuric acid at 0.5, 1.0 and 1.5% (w/v). A 10% solid loading was obtained by mixing 3.0 g of biomass with 30 mL of dilute acid in 100 mL serum bottles. Mixtures were autoclaved under standard liquid cycle (121 °C/15 psi) for residence times of 30, 45, or 60 min. The pretreatment intensity was maintained at low levels in this study to potentially reduce sugar degradation, the formation of inhibitors, such as furfurals, and also operating costs in scaled-up operations. Room-cooled, pretreated slurries were filtered through a porcelain Buchner funnel, and residual acid on the surface of pretreated biomass was washed with 250 mL of hot deionized water. Wet biomass left on the Buchner funnel was transferred to preweighed Ziplock plastic bags, weighed, and stored sealed at room temperature until use. Two 1.5 g subsets of the wet biomass were drawn from each pretreated sample and dried overnight in a 105 °C convection oven (Fisher Scientific) for solid recovery determination and a 40 °C vacuum oven (Fisher Scientific, model 48) for characterization of lignin and carbohydrate contents, respectively.

Enzymatic Hydrolysis. Six pretreatment conditions (two within each germplasm) based on lignin or hemicellulose solublization were selected for subsequent hydrolysis and fermentation. The three hydrolytic enzymes, cellulase (NS 50013, density of 1.20 g/mL), cellobiase (NS 50010, density of 1.24 g/mL), and xylanase (NS50030, density of 1.09 g/mL), were provided by Novozymes North America, Inc., Franklinton, NC. Cellulase and cellobiase activities measured by enzyme assays[17] were 75.5 filter paper units (FPU)/mL and 634.2 cellobiase units (CBU)/mL, respectively.

Hydrolysis was performed in 50 mL polypropylene centrifuge tubes in a water bath at 55 °C and 150 rpm for 72 h. Wet pretreated biomass samples equivalent to 1 g of dry matter were mixed with hydrolysis buffer containing 20 mL of 0.05 M citrate buffer (pH 4.8) and 40 μg/mL antibiotic, tetracycline hydrochloride. Hydrolyses of pretreated and unpretreated samples were performed at cellulase activities of 0, 15, and 30 FPU /g of dry biomass supplemented with cellobiase at an activity ratio of 4:1 CBU/FPU to avoid end-product inhibition because of cellobiose accumulation.[18,19] Xylanase at a loading of 0.25% (w/w) dry biomass was added to cellulase and cellobiase to test its effect on reducing sugar generation. The impact of background sugar contributed by enzymes (cellulase, xylanase, and cellobiase) was not accounted for in this study because of

(9) Esteghlalian, A.; Hashimoto, A. G.; Fenske, J. J.; Penner, M. H. Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass. *Bioresour. Technol.* **1997**, *59*, 129–136.

(10) Ballesteros, I.; Ballesteros, M.; Manzanares, P.; Negro, M. J.; Oliva, J. M.; Sáez, F. Dilute sulfuric acid pretreatment of cardoon for ethanol production. *Biochem. Eng. J.* **2008**, *42* (1), 84–91.

(11) Varga, E.; Réczey, K.; Zacchi, G. Optimization of steam pretreatment of corn stover to enhance enzymatic digestibility. *Appl. Biochem. Biotechnol.* **2004**, *113−116*, 509–523.

(12) Sun, Y.; Cheng, J. J. Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresour. Technol.* **2002**, *83*, 1–11.

(13) Wyman, C. E.; Dale, B. E.; Elander, R. T.; Holtzapple, M.; Ladisch, M. R.; Lee, Y. Y. Coordinated development of leading biomass pretreatment technologies. *Bioresour. Technol.* **2005**, *96*, 1959–1966.

(14) Torget, R.; Werdene, P.; Himmel, M.; Grohmann, K. Dilute acid pretreatment of short rotation woody and herbaceous crops. *Appl. Biochem. Biotechnol.* **1990**, *24/25*, 115–126.

(15) Burns, J. C.; Godshalk, E. B.; Timothy, D. H. Registration of "BoMaster" switchgrass. *J. Plant Registr.* **2008**, *2* (1), 31–32.

(16) Burns, J. C.; Godshalk, E. B.; Timothy, D. H. Registration of "Performer" switchgrass. *J. Plant Registr.* **2008**, *2* (1), 29–30.

(17) Ghose, T. K. Measurement of cellulase activities. *Pure Appl. Chem.* **1987**, *59*, 257–268.

(18) Ingesson, H.; Zacchi, G.; Yang, B.; Esteghlalian, A. R.; Saddler, J. N. The effect of shaking regime on the rate and extent of enzymatic hydrolysis of cellulose. *J. Biotechnol.* **2001**, *88*, 177–182.

(19) Bura, R.; Mansfield, S. D.; Saddler, J. N.; Bothast, R. J. SO_2-Catalyzed steam explosion of corn fiber for ethanol production. *Appl. Biochem. Biotechnol.* **2002**, *98−100*, 59–72.

negligible or low values reported by researchers.[20] Control samples (unpretreated or pretreated) soaked in hydrolysis buffer without enzymes (0 FPU/g of dry biomass) were used to study the effect of soaking on polysaccharide solublization. Hydrolyzates were centrifuged (model 5810R, Eppendorf) at 4 °C and 4000 rpm for 10 min, and 1 mL of supernatant was drawn, diluted, and filtered through 0.2 μm syringe filters for fermentable sugar analysis. Remaining hydrolyzates were autoclaved at 121 °C/15 psi for 15 min to inactivate contaminating microorganisms and degrade the antibiotic prior to fermentation.

Fermentation. *Saccharomyces cerevisiae* (ATCC 24859), cultured in a medium containing 20 g of glucose, 8.5 g of yeast extract, 1.32 g of NH_4Cl, 0.11 g of $MgSO_4$, and 0.06 g of $CaCl_2$ per liter of deionized water[21] was used. The cells were centrifuged and washed with 0.1% peptone water to remove residual media. The mean yeast inoculum count was 6.3×10^7 colony forming units (cfu)/mL corresponding to 11 g of dry cell weight/L.

A total of 250 μL of yeast inoculum was added to each tube containing 19 mL of sterile hydrolyzate for fermention in an incubator at 30 °C for 48 h. Fermented samples were centrifuged at 4 °C and 4000 rpm for 10 min; supernatant was filtered through 0.2 μm syringe filters for determination of ethanol and residual sugar contents.

Composition Analyses. Composition analyses of the unpretreated whole-plant and plant-part (stem and leaf) samples for each switchgrass germplasm (total of nine biomass samples) were performed in triplicate to determine total solids, ash, acid-insoluble lignin (AIL), acid-soluble lignin (ASL), and carbohydrates (primarily on the basis of glucan, xylan, and arabinan) according to the Laboratory Analytical Procedures (LAPs) of the National Renewable Energy Laboratory (NREL).[22−24] Extractives were analyzed with a method by Han and Rowell.[25] The LAP for carbohydrate analysis was modified to determine monomeric sugars (glucose, xylose, and arabinose) using a Shimadzu (Kyoto, Japan) high-performance liquid chromatography (HPLC) instrument 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, dried at 40 °C, was characterized for lignin (AIL and ASL) and carbohydrate contents (glucose, xylose, and arabinose). Solids remaining in the pretreated biomass were determined as total solids. Hydrolyzates were analyzed for reducing sugars (cellobiose, glucose, xylose, and arabinose), and fermentation broths were analyzed for residual reducing sugars and ethanol. Ethanol content in the fermentation broth was measured using the same HPLC procedure but including ethanol as a standard during calibration.

Percent hemicellulose solublization resulting from acid pretreatment was calculated as the ratio of the difference in xylose plus arabinose content of unpretreated and pretreated samples to the content in unpretreated samples. Hydrolysis efficiency was calculated by comparing glucose yield (in grams) after hydrolysis with glucose content (in grams or presented as 1.1 × the initial glucan content) of prehydrolysis biomass (unpretreated or pretreated), using eq 1,[11]

$$\text{glucan conversion efficiency} = \frac{C \times V \times a \times \dfrac{1\text{ L}}{1000\text{ mL}}}{m \times \%\text{ glucan} \times 1.1} \times 100\% \quad (1)$$

where C is the concentration of glucose after enzymatic hydrolysis detected by HPLC (g/L), V is the volume of the hydrolyzate (in milliliters), a is the the dilution rate of hydrolyzate before HPLC sugar analysis, m is the weight of dry biomass (unpretreated or pretreated) before hydrolysis (in grams), and "% glucan" was determined by carbohydrate analysis of the unpretreated or pretreated biomass.

Ethanol yield was calculated by dividing the total amount of ethanol produced (grams of ethanol) in the fermentation broth by the initial weight of switchgrass processed (in grams of unpretreated biomass). The percent ethanol yield relative to the theoretical yield was calculated using eq 2

$$\text{percent ethanol yield} = \frac{\text{ethanol (g)/biomass (g)}}{0.511 \times \text{glucose (g)/biomass (g)}} = E \times V \times d \times \frac{\dfrac{1\text{ L}}{1000\text{ mL}} \times \dfrac{\text{SR}}{100}}{0.511 \times \dfrac{G}{100}} \times 100\% \quad (2)$$

where 0.511 is the theoretical ethanol yield (in grams) generated per 1 g of glucose,[26] E is the concentration of ethanol after fermentation detected by HPLC (g/L), V is the volume of fermented hydrolysate (in milliliters), d is the dilution rate for HPLC analysis, SR is the % solid recovery after pretreatment, and G is the % glucose content in corresponding unpretreated biomass (obtained from Tables 2−4).

Statistical Analysis. Multiple comparison with the best[27] was performed using SAS (version 9.1.3, Cary, NC) to identify the most effective pretreatment conditions based on the least lignin content or greatest hemicellulose solublization for each germplasm. Triplicate data from nine pretreatment conditions (three acid concentrations × three residence times) for each whole-plant or plant-part sample of the three germplasms were used for this analysis. Additionally, analysis of variance (ANOVA) was performed using SAS (version 9.1.3, Cary, NC) to determine individual and interactive effects of the acid concentration and treatment time during pretreatment as well as cellulase activity and xylanase addition during hydrolysis/fermentation. Tukey simultaneous tests were performed to test differences among treatments. A 95% confidence level was applied to all analyses.

Results and Discussion

Composition of Switchgrass. Compositions of three freeze-dried switchgrass germplasms, each with one whole-plant and two plant-part samples (stems and leaves), are presented in Table 1. Besides a moisture content of 1.37−2.26%, samples contained 22.71−30.95% glucan, 13.27−18.58% xylan, 1.54−2.46% arabinan, and 17.39−20.60% lignin db. Stem samples were similar in composition to whole plants, possibly because of a greater proportion of stem than leaf in the whole plant. It has

(20) O'Dwyer, J. P.; Zhu, L.; Granda, C. B.; Holtzapple, M. T. Enzymatic hydrolysis of lime-pretreated corn stover and investigation of the HCH-1 model: Inhibition pattern, degree of inhibition, validity of simplified HCH-1 model. *Bioresour. Technol.* **2007**, *98*, 2969–2977.

(21) Chen, Y.; Sharma-Shivappa, R. R.; Keshwani, D.; Chen, C. Potential of agricultural residues and hay for bioethanol production. *Appl. Biochem. Biotechnol.* **2007**, *142*, 276–290.

(22) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. Determination of total solids in biomass. In *Laboratory Analytical Procedures*; National Renewable Energy Laboratory: Golden, CO, 2005.

(23) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. Determination of ash in biomass. In *Laboratory Analytical Procedures*; National Renewable Energy Laboratory: Golden, CO, 2005.

(24) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. Determination of structural carbohydrates and lignin in biomass. In *Laboratory Analytical Procedures*; National Renewable Energy Laboratory: Golden, CO, 2006.

(25) Han, J.; Rowell, J. *Chemical Compositions of Agro-Based Fibers. Paper and Composites from Agro-Based Resources*; CRC Press: Boca Raton, FL, 1996.

(26) Thomas, K. C.; Hynes, S. H.; Ingledew, W. M. Practical and theoretical considerations in the production of high concentrations of alcohol by fermentation. *Process Biochem.* **1996**, *31* (4), 321–331.

(27) Hsu, J. C. *Multiple Comparisons Theory and Methods*; Chapman and Hall: London, U.K., 1996.

Table 1. Composition of Three Freeze-Dried Switchgrass Germplasms and Their Plant Parts (Average Expressed on a Percent Dry Matter Basis with Standard Deviations in Parentheses; $N = 3$)

	germplasm and plant part								
	St6-1			St6-3E			St6-3F		
composition	whole	stem	leaf	whole	stem	leaf	whole	stem	leaf
ash	2.63 (0.05)	2.39 (0.02)	3.97 (0.00)	2.99 (0.03)	2.30 (0.01)	4.15 (0.01)	2.98 (0.02)	2.66 (0.02)	4.72 (0.01)
extractives	4.05 (0.20)	5.25 (0.54)	4.33 (0.63)	6.43 (0.65)	5.33 (0.12)	6.65 (2.17)	6.92 (0.57)	7.41 (0.85)	9.00 (0.78)
AIL	17.14 (0.31)	18.45 (0.28)	16.99 (0.36)	15.32 (0.16)	16.06 (0.48)	13.18 (0.87)	14.58 (0.05)	15.46 (0.25)	13.83 (0.99)
ASL	2.32 (0.04)	1.95 (0.02)	3.61 (0.03)	2.61 (0.19)	2.11 (0.07)	4.59 (0.26)	2.81 (0.12)	2.35 (0.05)	4.07 (0.23)
glucan	30.95 (0.94)	30.85 (2.17)	23.21 (0.86)	28.68 (2.82)	27.50 (0.75)	24.70 (2.02)	24.34 (1.63)	24.30 (1.43)	22.71 (2.18)
xylan	18.58 (0.50)	18.06 (1.29)	14.82 (0.49)	18.11 (1.94)	17.47 (0.49)	15.01 (1.45)	14.68 (0.95)	15.15 (0.96)	13.27 (1.24)
arabinan	2.15 (0.16)	1.95 (0.28)	2.34 (0.16)	2.14 (0.15)	1.75 (0.00)	2.46 (0.25)	2.00 (0.15)	1.54 (0.31)	2.40 (0.26)
others	22.18	21.10	30.73	23.72	27.48	29.26	31.69	31.13	30.00

been reported that the leaf/stem ratio of perennial grasses changes (decreases) as grasses mature.[28] Ash and extractives were generally greater in the leaf than in the stem, and it was also observed that St6-3F had a greater fraction of these two components than St6-1 and St6-3E. However, ash and extractives in the three germplasms were less than those in commercial switchgrass cultivars, such as Alamo and Cave-in-Rock, as reported in the biomass feedstock composition and property database;[29] specifically, extractives were approximately 50% of the value presented in the database. These variations may be due to varietal differences, and difficulties in achieving complete extraction as extractives in lignocellulosic feedstocks are made up of diverse materials, such as waxes, fats, gums, starches, resins, and essential oils.[30] In addition, the efficiency of an extraction process is impacted by external factors, such as condenser temperature and siphon rate, during extraction.[31]

AIL, which provides strength and protection for the plant,[32] formed a predominant portion of the total lignin in switchgrass germplasms studied. Although total lignin content was 18−20% on a dry matter basis in all switchgrass samples, the stem contained greater AIL and less ASL than the leaf. Lignin is known to accumulate as the stem elongates, and elevated lignin content indicates increased maturity and reduced whole-plant digestibility.[33] When germplasms are compared, the total lignin in St6-1 was greater than that in St6-3E and St6-3F. The low lignin content of St6-3F, especially the AIL, may be correlated with its improved digestibility characteristic.

Glucan and xylan, at a ratio of 3:2, were two of the most abundant polysaccharides in switchgrass and contributed 40−50% of the dry matter. St6-1 and St6-3F contained the greatest and least sugar content, respectively. Within each germplasm, the sugar content in the stem was similar to that in the whole plant, whereas leaves contained less glucan and xylan but slightly greater arabinan. Although leaves contain smaller quantities of cell-wall polysaccharides than the stem, improved plant digestibility can be achieved with a higher leaf/stem ratio.[34] Components such as uronic acid, acetyl groups, and minerals[10,35−37] that may be present in switchgrass were not quantified in this study. Because the composition of switchgrass depends upon factors such as harvest time and maturity level,[38,39] certain compositional variances within germplasms and relative to other varieties were considered reasonable.

Pretreatment. Sulfuric acid pretreatment resulted in 52.13−78.23% solid recovery of switchgrass (Tables 2−4). Germplasm, type of plant sample, treatment time, and acid concentration significantly ($p < 0.05$) affected solid recovery, with the effect of acid concentration being greatest based on the associated F value. Generally, solid recovery decreased as the acid concentration and treatment time increased. Relative to plant parts, stem samples gave the greatest solid recovery, while leaves were most susceptible to acid pretreatment.

Effect of Pretreatment on Lignin Content. Lignin is very sensitive to sulfuric acid even under mild conditions, and both degradation and accumulation can occur simultaneously when lignin and acid interact.[40,41] Acid pretreatment conditions under which delignification predominates accumulation have been discussed in other studies.[42,43] Dilute

(28) Stone, B. A. Prospects for improving the nutritive value of temperature, perennial pasture grasses. *N. Z. J. Agric. Res.* **1994**, *37*, 349–363.
(29) U.S. Department of Energy, Energy Efficiency and Renewable Energy. Biomass feedstock composition and property database (available online at http://www1.eere.energy.gov/biomass/feedstock_databases.html.
(30) Kuhad, R. C.; Singh, A. Lignocellulose biotechnology: Current and future prospects. *Crit. Rev. Biotechnol.* **1993**, *13* (2), 151–172.
(31) Sluiter, A.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. Determination of extractives in biomass. In *Laboratory Analytical Procedures*; National Renewable Energy Laboratory: Golden, CO, 2005.
(32) Brett, C. T.; Waldron, K. W. *Physiology and Biochemistry of Plant Cell Walls*, 2nd ed.; Chapman and Hall: London, U.K., 1996.
(33) Walker, D. W.; West, C. P.; Bacon, R. K.; Longer, D. E.; Turner, K. E. Changes in forage yield and composition of wheat and wheat−ryegrass mixtures with maturity. *J. Dairy Sci.* **1990**, *73*, 1296–1303.
(34) Bourquin, L. D.; Fahey, G. C. Ruminal digestion and glycosyl linkage patterns of cell wall components from leaf and stem fractions of alfalfa, orchardgrass, and wheat straw. *J. Anim. Sci.* **1994**, *72*, 1362–1374.
(35) Kaar, W. E.; Holtzapple, M. T. Using lime pretreatment to facilitate the enzymatic hydrolysis of corn stover. *Biomass Bioenergy* **2000**, *18*, 189–199.
(36) Samson, R.; Mehdi, B. Strategies to reduce the ash content in perennial grasses. Research report for resource efficient agricultural production, Quebec, Canada, 1998.
(37) Samson, R.; Mani, S.; Boddey, R.; Sokhansanj, S.; Quesada, D.; Urquiaga, S.; Reis, V.; Ho Lem, C. The potential of C_4 perennial grasses for developing a global bioheat industry. *Crit. Rev. Plant Sci.* **2005**, *24*, 461–495.
(38) Adler, P. R.; Sanderson, M. A.; Boateng, A. A.; Weimer, P. J.; Jung, H.-J. G. Biomass yield and biofuel quality of switchgrass harvested in fall or spring. *Agron. J.* **2006**, *98* (6), 1518–1525.
(39) Dien, B. S.; Jung, H.-J. G.; Vogel, K. P.; Casler, M. D.; Lamb, J. F. S.; Iten, L.; Mitchell, R. B.; Sarath, G. Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass. *Biomass Bioenergy* **2006**, *30*, 880–891.
(40) Pearl, I. A. *The Chemistry of Lignin*; Marcel Dekker, Inc.: New York, 1967.
(41) Sjostrom, E. *Wood Chemistry*; Academic Press, Inc.: New York, 1993.
(42) Sarkanen, K. V.; Ludwig, C. H. *Lignins: Occurrence, Formation, Structure and Reactions*; John Wiley and Sons, Inc.: New York, 1971.
(43) Liao, W.; Liu, Y.; Wen, Z.; Frear, C.; Chen, S. Studying the effects of reaction conditions on components of dairy manure and cellulose accumulation using dilute acid treatment. *Bioresour. Technol.* **2007**, *98*, 1992–1999.

Table 2. Composition of St6-1 Freeze-Dried Switchgrass after Acid Pretreatment at Different Conditions (Expressed as g/100 g of Unpretreated Dry Switchgrass; $N = 27$)

	pretreatment conditions				composition of solid fractions[a] (%)			
plant (part)	time (min)	acid concentration (%)	total solid[a]	hemicellulose[a] solublization (%)	lignin	glucose	xylose	arabinose
whole	unpretreated sample		100.00		19.48 (0.28)	34.05 (1.03)	20.44 (0.55)	2.37 (0.18)
	30	0.5	73.35 (0.46)	39.68 (18.55)	19.82 (0.48)	33.35 (10.73)	13.11 (3.85)	0.65 (0.38)
		1.0	65.93 (1.13)	55.81 (3.82)	18.80 (0.16)	36.18 (3.39)	9.59 (0.77)	0.49 (0.12)
		1.5	64.06 (1.67)	63.50 (11.66)	19.44 (0.53)	29.84 (0.91)	8.22 (2.75)	0.11 (0.15)
	45	0.5	69.92 (0.74)	43.36 (5.64)	18.90 (0.19)	33.05 (1.91)	12.29 (1.16)	0.63 (0.13)
		1.0	63.97 (0.56)	69.70 (3.63)	18.34 (0.39)[b]	27.97 (3.04)	6.51 (0.82)	0.40 (0.01)
		1.5	60.07 (0.10)	74.53 (5.30)	18.59 (0.12)	29.33 (4.26)	5.48 (1.11)	0.33 (0.11)
	60	0.5	67.75 (2.65)	33.27 (4.54)	18.82 (0.00)	38.81 (3.66)	14.65 (1.23)	0.58 (0.19)
		1.0	61.08 (0.22)	73.22 (1.11)	18.65 (0.03)	31.62 (0.35)	5.83 (0.19)	0.27 (0.06)
		1.5	57.93 (0.96)	77.69 (2.59)	20.93 (0.19)	32.93 (3.68)	4.86 (0.74)	0.22 (0.16)
stem	unpretreated sample		100.00		20.41 (0.30)	33.93 (2.39)	19.87 (1.42)	2.14 (0.31)
	30	0.5	74.72 (1.11)	47.43 (4.15)	20.76 (0.15)	30.86 (3.76)	11.07 (0.93)	0.50 (0.01)
		1.0	68.89 (1.92)	58.80 (12.67)	19.59 (0.06)	32.44 (9.73)	8.55 (2.65)	0.51 (0.13)
		1.5	66.15 (1.03)	60.32 (5.18)	19.89 (0.29)	36.65 (1.71)	8.35 (1.12)	0.38 (0.03)
	45	0.5	73.50 (0.33)	44.56 (1.58)	19.89 (0.18)	31.48 (0.98)	11.71 (0.35)	0.49 (0.01)
		1.0	67.33 (0.73)	67.66 (0.81)	19.62 (0.13)	29.23 (1.99)	6.69 (0.19)	0.42 (0.01)
		1.5	64.59 (0.61)	73.84 (2.80)	19.55 (0.27)	29.82 (2.51)	5.44 (0.47)	0.31 (0.15)
	60	0.5	71.37 (1.19)	41.95 (6.04)	18.47 (1.27)	39.28 (4.63)	12.31 (1.34)	0.46 (0.01)
		1.0	63.49 (0.93)	73.36 (1.72)	19.72 (0.40)	29.44 (0.95)	5.58 (0.32)	0.29 (0.06)
		1.5	61.24 (1.32)	75.09 (1.00)	21.30 (0.04)	36.50 (1.61)	5.26 (0.05)	0.22 (0.18)
leaf	unpretreated sample		100.00		20.60 (0.39)	25.53 (0.95)	16.30 (0.54)	2.57 (0.18)
	30	0.5	70.70 (1.09)	37.97 (12.62)	22.54 (0.18)	29.68 (6.23)	10.92 (2.13)	0.78 (0.27)
		1.0	63.03 (0.47)	75.05 (3.58)	21.51 (0.13)	18.82 (1.38)	4.43 (0.58)	0.28 (0.11)
		1.5	60.09 (2.56)	71.70 (10.38)	21.54 (0.11)	26.53 (3.38)	5.10 (1.82)	0.23 (0.23)
	45	0.5	74.57 (0.58)	30.02 (2.25)	21.80 (0.20)	24.74 (0.86)	12.46 (0.43)	0.74 (0.00)
		1.0	62.69 (0.75)	78.21 (6.79)[c]	21.31 (0.20)	19.03 (6.14)	3.91 (1.28)	0.20 (0.00)
		1.5	59.34 (0.37)	76.97 (0.40)	21.13 (0.24)	24.59 (0.45)	4.15 (0.07)	0.19 (0.00)
	60	0.5	71.43 (1.15)	19.32 (12.46)	21.39 (0.07)	34.84 (4.33)	14.21 (2.19)	1.01 (0.16)
		1.0	58.92 (0.37)	77.44 (3.94)	21.12 (0.15)	22.90 (2.83)	4.05 (0.64)	0.20 (0.10)
		1.5	57.14 (0.80)	73.30 (0.42)	21.86 (0.97)	33.32 (1.60)	4.75 (0.20)	0.29 (0.13)

[a] Expressed as mean value (standard deviation). [b] The least lignin availability observed for this germplasm after pretreatment. [c] The greatest hemicellulose solublization observed for this germplasm after pretreatment.

acid pretreatment of dairy manure resulted in lignin accumulation, which was greater than lignin degradation at all selected pretreatment conditions of 110−130 °C, 1−3 h, and 1−3% acid.[43] However, in this study, acid pretreatment of switchgrass resulted in both lignin degradation and accumulation, with accumulation being more predominant in the treated samples.

The greatest lignin removal was limited to 9.51, 6.99, and 8.28% for St6-1 stem, St6-3E stem, and St6-3F whole plant, respectively (Tables 2−4). Lignin accumulation (9.42−21.40%) was usually observed when pretreating leaf samples with the most dilute acid (0.5% H_2SO_4) for the shortest residence time (30 min). Agblevor et al.[44] found that organic and inorganic materials present in the biomass can condense with lignin during composition analysis with 72% H_2SO_4 treatment and these acid-insoluble materials may cause an over estimation of the actual lignin content. Because switchgrass leaf samples contained the greatest extractives, which may not have been completely removed during mild acid pretreatment, it is possible that the extractives condensed with lignin to form acid-insoluble materials and resulted in the greater values of AIL. Another factor possibly contributing to over estimation of total lignin is the presence of components other than acid-soluble lignin that can absorb light at a wavelength of 205 nm during spectrophotometeric ASL analysis.[45]

Although little lignin degradation was observed during acid pretreatment, the type of plant sample (whole, stem, or leaf), acid concentration, residence time, and the interaction between acid and time all had significant ($p < 0.05$) influence on lignin content in pretreated samples within each switchgrass germplasm.

Effect of Pretreatment on Hemicellulose Solublization. Because hemicellulose interaction with cellulose forms a physical barrier to enzyme attack, effective hemicellulose solublization can help enhance digestibility of cellulose, thus increasing enzymatic conversion efficiency during hydrolysis.[46−48] Unlike lignin, hemicellulose (both xylan and arabinan) solublization was enhanced by increasing the acid concentration and extending the residence time. The interaction between concentration and time had a significant

(44) Agblevor, F. A.; Batz, S.; Trumbo, J. Composition and ethanol production potential of cotton gin residues. *Appl. Biochem. Biotechnol.* **2003**, *105*−*108*, 219–230.

(45) Ehrman, T. Determination of acid-soluble lignin in biomass. In *Laboratory Analytical Procedure LAP-004*; National Renewable Energy Laboratory: Golden, CO, 1996.

(46) Hsu, T.-A. Pretreatment of biomass. In *Handbook on Bioethanol: Production and Utilization*; Taylor and Francis: Washington, D.C., 1996.

(47) Mosier, N.; Wyman, C.; Dale, B.; Elander, R.; Lee, Y. Y.; Holtzapple, M.; Ladisch, M. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* **2005**, *96*, 673–686.

(48) Kabel, M. A.; Bos, G.; Zeevalking, J.; Voragen, A. G. J.; Schols, H. A. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. *Bioresour. Technol.* **2007**, *98*, 2034–2042.

Table 3. Composition of St6-3E Freeze-Dried Switchgrass after Acid Pretreatment at Different Conditions (Expressed as g/100 g of Unpretreated Dry Switchgrass; $N = 27$)

pretreatment conditions					composition of solid fractions[a] (%)			
plant (part)	time (min)	acid concentration (%)	total solid[a]	hemicellulose[a] solublization (%)	lignin	glucose	xylose	arabinose
whole	unpretreated sample		100.00		17.93 (0.24)	31.55 (3.10)	19.92 (2.13)	2.35 (0.16)
	30	0.5	71.24 (1.22)	57.09 (15.56)	19.68 (0.58)	22.46 (9.24)	9.16 (3.21)	0.39 (0.26)
		1.0	65.40 (1.11)	57.02 (8.04)	19.05 (0.30)	31.25 (5.90)	8.93 (1.58)	0.64 (0.21)
		1.5	62.57 (0.25)	69.96 (6.13)	19.00 (0.64)	26.37 (6.73)	6.35 (1.26)	0.34 (0.11)
	45	0.5	71.06 (1.27)	52.37 (8.42)	18.83 (0.36)	29.95 (2.74)	10.06 (1.84)	0.55 (0.13)
		1.0	61.50 (1.01)	65.91 (1.97)	18.70 (0.11)	30.16 (1.78)	7.11 (0.32)	0.48 (0.11)
		1.5	57.58 (1.34)	79.45 (0.64)	18.08 (0.06)	25.06 (2.09)	4.37 (0.13)	0.20 (0.01)
	60	0.5	71.68 (4.42)	39.95 (16.63)	19.06 (0.31)	35.26 (6.68)	12.65 (3.42)	0.72 (0.29)
		1.0	59.09 (0.60)	72.86 (1.42)	18.64 (0.11)	32.56 (2.47)	5.75 (0.32)	0.30 (0.00)
		1.5	57.52 (0.98)	80.39 (3.91)	19.44 (0.51)	30.86 (7.08)	4.26 (1.00)	0.11 (0.12)
stem	unpretreated sample		100.00		18.17 (0.55)	30.25 (0.82)	19.22 (0.54)	1.92 (0.00)
	30	0.5	73.15 (0.49)	56.57 (4.40)	19.25 (0.35)	20.59 (2.17)	8.85 (0.79)	0.33 (0.14)
		1.0	66.95 (0.56)	54.21 (9.63)	18.32 (0.41)	31.71 (5.94)	9.11 (1.93)	0.57 (0.12)
		1.5	63.44 (1.07)	67.46 (4.83)	18.18 (0.25)	25.03 (3.81)	6.54 (0.91)	0.34 (0.11)
	45	0.5	69.89 (0.46)	38.69 (7.58)	18.35 (0.04)	30.64 (4.02)	12.33 (1.49)	0.63 (0.13)
		1.0	63.31 (1.08)	62.60 (2.06)	18.09 (0.29)	30.69 (1.11)	7.49 (0.43)	0.42 (0.00)
		1.5	59.95 (0.43)	74.33 (1.24)	18.68 (0.56)	28.37 (1.74)	5.04 (0.27)	0.38 (0.01)
	60	0.5	70.11 (5.05)	27.25 (21.03)	18.14 (0.58)	40.12 (4.40)	14.67 (4.18)	0.71 (0.28)
		1.0	59.85 (0.51)	73.71 (1.11)	16.90 (0.89)[b]	23.93 (0.74)	5.29 (0.21)	0.27 (0.06)
		1.5	59.44 (1.45)	78.50 (3.30)	18.50 (0.68)	29.31 (5.37)	4.29 (0.68)	0.26 (0.01)
leaf	unpretreated sample		100.00		17.76 (1.13)	27.17 (2.22)	16.51 (1.59)	2.71 (0.27)
	30	0.5	78.23 (1.60)	19.22 (2.46)	21.50 (0.31)	26.93 (1.46)	14.25 (0.30)	1.27 (0.17)
		1.0	60.42 (0.62)	69.73 (12.59)	19.38 (0.20)	21.00 (7.94)	5.42 (2.23)	0.40 (0.20)
		1.5	57.76 (0.14)	76.12 (7.05)	20.01 (0.39)	20.90 (5.11)	4.34 (1.30)	0.25 (0.11)
	45	0.5	72.71 (0.49)	36.60 (7.33)	20.27 (0.11)	24.96 (1.74)	11.30 (1.15)	0.89 (0.28)
		1.0	60.87 (3.71)	70.25 (1.19)	19.20 (0.05)	25.07 (1.23)	5.38 (0.15)	0.34 (0.11)
		1.5	54.89 (0.53)	76.62 (2.16)	19.92 (0.10)	26.42 (0.79)	4.20 (0.31)	0.30 (0.10)
	60	0.5	61.58 (4.59)	48.28 (10.96)	19.80 (0.30)	32.93 (2.62)	9.24 (2.12)	0.70 (0.18)
		1.0	55.34 (0.41)	74.92 (1.99)	19.44 (0.25)	26.89 (2.25)	4.50 (0.33)	0.32 (0.05)
		1.5	53.05 (0.16)	83.99 (2.95)[c]	19.72 (0.38)	23.72 (3.65)	2.91 (0.52)	0.17 (0.04)

[a] Expressed as mean value (standard deviation). [b] The least lignin availability observed for this germplasm after pretreatment. [c] The greatest hemicellulose solublization observed for this germplasm after pretreatment.

effect ($p < 0.05$) on hemicellulose solublization for all three germplasms. For St6-3F, the type of plant sample and treatment time were also critical ($p < 0.05$) for predicting the degree of hemicellulose solublization.

Percent hemicellulose solublization in pretreated samples ranged from 19.32 to 78.21 for St6-1, from 19.22 to 83.99 for St6-3E, and from 36.81 to 85.87 for St6-3F (Tables 2−4). The higher level of hemicellulose solublization in St6-3F may be attributed to its improved digestibility. Solublization observed for leaf samples was greater than stem. Approximately 80% of the hemicellulose could be removed from the lignocellulosic matrix by pretreatment using 1.5% acid for 60 min. Hemicellulose in the lignocellulosic feedstock can be completely solublized through a two-stage dilute sulfuric acid percolation (DA) process.[21,49] At temperatures above 121 °C, greater hemicellulose solublization (>90%) can be achieved within a short time period (<10 min).[9,14] The hemicellulose solubilized during acid pretreatment of the switchgrass samples was separated from the residual solid fractions by filtration. Although the filtrate was not analyzed, similar analysis during pretreatment of oven-dried switchgrass samples resulted in xylose-rich filtrate.[51]

Effect of Pretreatment on Other Cell-Wall Components. At least 70% of the cellulose (on the basis of glucose analysis) in the pretreated residual solids was recovered. One exception was 60.4% cellulose recovery in St6-3F leaf samples after being pretreated by 1.0% acid for 60 min. The glucose content in pretreated samples decreased as treatment conditions intensified relative to the acid concentration and treatment time. Some pretreated samples had glucose content higher than unpretreated samples, which may be attributed to heterogeneity in biomass composition and measurement errors. The type of plant sample and treatment time significantly ($p < 0.05$) affected glucose content after pretreatment for all three germplasms. Although the acid concentration had a significant ($p < 0.05$) effect on glucose content in St6-1 and St6-3F, the interaction term between acid concentration and time was not significant ($p \geq 0.05$) for the pretreated St6-1 switchgrass samples.

Through post-pretreatment composition analyses (Table 2−4), lignin and sugars present in pretreated samples added up more closely to the total recovered solids as treatment conditions became more intense. Therefore, pretreatments may have varying impacts on other unquantified plant cell components, such as proteins, ash, and extractives. Proteins decrease as the acid concentration, reaction temperature, and time increase,[43] while certain extractives can be removed

(49) Wu, Z.; Lee, Y. Ammonia recycled percolation as a complementary pretreatment to the dilute-acid process. *Appl. Biochem. Biotechnol.* **1997**, *63*, 21–34.

(50) Oh, K. K.; Kim, Y. S.; Yoon, H. H.; Tae, B. S. Pretreatment of lignocellulosic biomass using combination of ammonia recycled percolation and dilute-acid process. *J. Ind. Eng. Chem.* **2002**, *8* (1), 64–70.

(51) Yang, Y.; Sharma-Shivappa, R. R.; Burns, J. C.; Cheng, J. J. Dilute acid pretreatment of oven-dried switchgrass germplasms for bioethanol production. *Energy Fuels* **2009**, *23*, 3759–3766.

Table 4. Composition of St6-3F Freeze-Dried Switchgrass after Acid Pretreatment at Different Conditions (Expressed as g/100 g of Unpretreated Dry Switchgrass; $N = 27$)

pretreatment conditions					composition of solid fractions[a] (%)			
plant (part)	time (min)	acid concentration (%)	total solid[a]	hemicellulose[a] solublization (%)	lignin	glucose	xylose	arabinose
whole		unpretreated sample	100.00		17.39 (0.08)	26.77 (1.79)	16.15 (1.05)	2.20 (0.17)
	30	0.5	71.91 (0.40)	36.81 (7.49)	18.66 (0.50)	22.18 (2.15)	11.02 (1.26)	0.57 (0.12)
		1.0	60.24 (1.20)	59.51 (6.14)	16.17 (0.24)	24.98 (3.61)	6.98 (1.04)	0.45 (0.09)
		1.5	57.93 (0.11)	67.31 (2.58)	16.72 (0.38)	25.75 (3.06)	5.62 (0.46)	0.38 (0.02)
	45	0.5	63.94 (1.35)	57.14 (5.55)	16.06 (0.17)	20.30 (2.14)	7.51 (0.90)	0.35 (0.12)
		1.0	58.02 (0.69)	70.82 (4.60)	16.08 (0.07)	21.63 (2.82)	5.10 (0.73)	0.26 (0.12)
		1.5	55.77 (1.09)	75.41 (2.00)	15.95 (0.23)[b]	23.58 (2.17)	4.33 (0.36)	0.19 (0.00)
	60	0.5	59.80 (2.08)	76.56 (2.67)	18.63 (0.38)	18.87 (1.62)	4.10 (0.48)	0.20 (0.01)
		1.0	59.50 (0.47)	78.01 (1.72)	16.86 (0.14)	21.46 (1.02)	3.84 (0.32)	0.19 (0.00)
		1.5	54.23 (0.45)	75.75 (0.87)	17.22 (0.26)	31.07 (1.08)	4.20 (0.15)	0.25 (0.01)
stem		unpretreated sample	100.00		17.81 (0.22)	26.73 (1.57)	16.67 (1.06)	1.69 (0.34)
	30	0.5	76.76 (0.28)	40.87 (22.53)	19.50 (0.08)	19.76 (6.92)	10.32 (3.88)	0.54 (0.27)
		1.0	64.89 (0.34)	60.49 (9.09)	17.48 (0.25)	23.36 (5.15)	6.90 (1.56)	0.35 (0.12)
		1.5	62.75 (0.48)	56.81 (11.95)	17.48 (0.14)	30.52 (7.29)	7.47 (2.09)	0.46 (0.11)
	45	0.5	70.77 (0.43)	44.47 (3.18)	18.56 (0.26)	22.73 (1.58)	9.74 (0.60)	0.46 (0.02)
		1.0	62.20 (0.85)	57.41 (3.16)	17.79 (0.24)	31.42 (3.05)	7.42 (0.58)	0.40 (0.00)
		1.5	60.18 (0.89)	67.57 (1.93)	18.34 (0.34)	29.12 (1.66)	5.56 (0.35)	0.39 (0.01)
	60	0.5	64.28 (0.58)	69.75 (7.30)	18.88 (0.25)	19.79 (3.77)	5.27 (1.21)	0.29 (0.13)
		1.0	59.78 (0.90)	78.57 (0.35)	17.81 (0.08)	19.20 (1.81)	3.73 (0.06)	0.20 (0.00)
		1.5	57.49 (1.12)	78.60 (4.07)	17.64 (0.29)	26.49 (4.97)	3.78 (0.67)	0.15 (0.13)
leaf		unpretreated sample	100.00		17.90 (1.22)	24.98 (2.40)	14.60 (1.36)	2.64 (0.29)
	30	0.5	77.80 (0.34)	38.51 (5.99)	21.73 (0.29)	18.66 (2.03)	9.75 (1.02)	0.85 (0.03)
		1.0	59.20 (0.59)	78.62 (3.36)	18.19 (0.10)	16.74 (2.24)	3.50 (0.58)	0.19 (0.00)
		1.5	56.76 (0.52)	72.06 (9.59)	18.38 (0.46)	22.84 (6.58)	4.51 (1.54)	0.31 (0.11)
	45	0.5	59.30 (0.44)	61.25 (11.75)	16.08 (0.45)	16.97 (2.16)	6.10 (1.85)	0.58 (0.19)
		1.0	57.46 (0.36)	68.82 (2.51)	17.85 (1.22)	25.24 (1.70)	5.06 (0.33)	0.32 (0.11)
		1.5	54.24 (0.15)	78.35 (2.03)	19.09 (0.28)	23.54 (3.01)	3.55 (0.35)	0.18 (0.00)
	60	0.5	66.96 (6.03)	67.32 (3.88)	20.09 (0.34)	17.70 (2.36)	5.33 (0.74)	0.30 (0.12)
		1.0	55.11 (0.20)	85.87 (0.16)[c]	18.46 (0.15)	15.09 (0.26)	2.25 (0.02)	0.19 (0.00)
		1.5	52.13 (0.59)	85.43 (1.47)	18.66 (0.37)	22.17 (2.37)	2.46 (0.30)	0.05 (0.06)

[a] Expressed as mean value (standard deviation). [b] The least lignin availability observed for this germplasm after pretreatment. [c] The greatest hemicellulose solublization observed for this germplasm after pretreatment.

simultaneously with hemicellulose during dilute sulfuric acid pretreatment.[52] Relative to ash, acid pretreatment does not impact ash content in pretreated switchgrass.[53]

Enzymatic Hydrolysis. Hydrolysis was conducted on two sets of pretreated samples from each germplasm based on the least percent lignin content and greatest percent hemicellulose solublization, irrespective of whether they were whole, stem, or leaf. Because plant parts can vary in their response to processing due to differences in composition, studying individual parts provides information on how suitable a particular component is for ethanol production; therefore, breeding efforts may be targeted toward enhancing the accumulation of the specific part and increasing its relative proportion in the whole plant.

Hydrolysis of the Pretreated Least Lignin Content Biomass. Pretreatment conditions that resulted in the least lignin content based on statistical analysis were 45 min and 1.0% acid for St6-1 whole plant, 60 min and 1.0% acid for St6-3E stem, and 45 min and 1.5% acid for St6-3F whole plant. The percent cellulose conversions obtained at various enzyme loadings during hydrolysis are summarized in Figure 1a.

Cellulose conversion in pretreated samples with the least amount of lignin ranged between 34.62 and 61.18% compared to 11.04 and 39.01% in unpretreated biomass. The greatest cellulose conversion observed was limited to 61% possibly because of the overall low degree of delignification ($< 10\%$), which can decrease the accessibility of the biomass to enzymes.

No cellobiose was detected in the hydrolyzates, indicating that cellobiose produced during hydrolysis was effectively hydrolyzed by cellobiase, therefore preventing end-product inhibition of cellulase. Of the various factors impacting hydrolysis, including (1) pretreatment, (2) cellulase addition and loading (0, 15, and 30 FPU/g of dry biomass), (3) addition of xylanase, and (4) interaction between cellulase loading and the presence of xylanase (FPU × xylanase), it was found that cellulase addition significantly ($p < 0.05$) affected percent cellulose conversion for all germplasms. Pretreatment was a significant factor for St6-3F samples, while enzyme interaction between cellulase loading and the presence of xylanase was significant ($p < 0.05$) for St6-1 samples. Although cellulase addition can greatly improve hydrolysis of cellulosic substrates, significant differences in cellulose conversion at 15 and 30 FPU/g of dry biomass were not observed ($p \geq 0.05$), except for the St6-1 whole plant.

Although the majority of the hemicellulose may have been removed from acid-pretreated biomass, the inhibitory effect of residual hemicellulose on cellulose can act as a physical barrier to cellulose conversion.[54] Therefore, xylanase

(52) Nguyen, Q. A.; Tucker, M. P.; Keller, F. A.; Eddy, F. P. Two-state dilute-acid pretreatment of softwoods. *Appl. Biochem. Biotechnol.* **2000**, *84*−*86*, 561–576.

(53) Chung, Y. C.; Bakalinsky, A.; Penner, M. H. Enzymatic saccharification and fermentation of xylose-optimized dilute acid-treated lignocellulosics. *Appl. Biochem. Biotechnol.* **2005**, *121*−*124*, 947–961.

(54) Öhgren, K.; Bura, R.; Saddler, J.; Zacchi, G. Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. *Bioresour. Technol.* **2007**, *98*, 2503–2510.

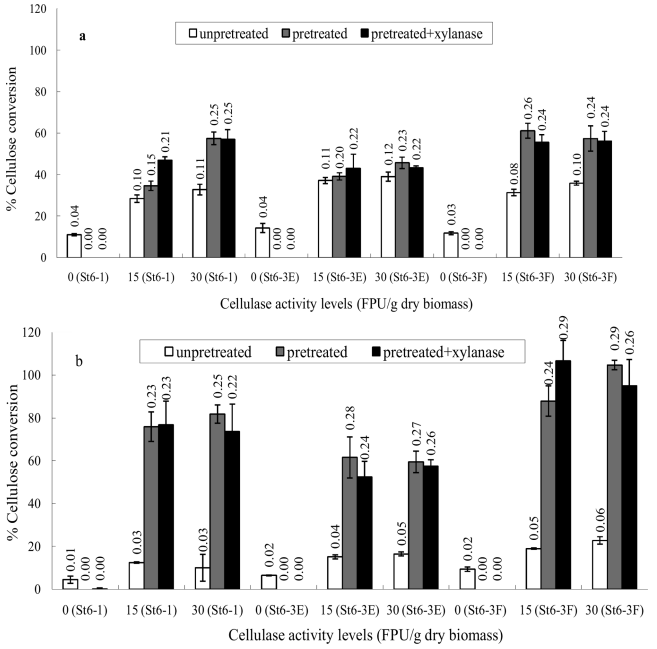

Figure 1. Hydrolysis of unpretreated and pretreated freeze-dried switchgrass plant (part) samples with the (a) least lignin content after pretreatment and (b) greatest hemicellulose solublization after pretreatment. Values on top of the columns indicate grams of glucose released during hydrolysis per gram of dry unpretreated or pretreated switchgrass.

supplementation can be beneficial to cellulose hydrolysis by reducing such inhibition, especially in samples pretreated by non-acidic methods that contain greater hemicellulose fractions. In this study, the addition of xylanase alone did not improve ($p \geq 0.05$) cellulose hydrolysis efficiency, although it significantly ($p < 0.05$) improved cellulose conversion from 34.62 to 46.98% at a cellulase loading of 15 FPU/g for St6-1 samples. Cellulase used in this study was a mixture of enzymes that may have contained xylanase activity[55] sufficient for hydrolyzing residual hemicelluloses.

Cellulose conversion ranging from 11.04 to 14.28% was observed in unpretreated switchgrass soaked in citrate buffer without cellulolytic or xylolytic enzymes. However, in buffer from pretreated control samples without enzymes, no monomeric sugars (glucose, xylose, and arabinose) were detected. Potentially, this was due to the transfer of free monomeric sugars from the biomass to the pretreatment liquid and then to the filtrate by washing of residual solids after dilute acid pretreatment. Thus, no free monomeric sugars were available for release from the surface of the pretreated samples during hydrolysis. Hence, for the 0 FPU unpretreated samples, percent cellulose conversion actually represents monomeric sugars that did not have to be enzymatically generated but contributed to the pool of sugars in the hydrolysate.

Hydrolysis of the Pretreated Greatest Hemicellulose Solublization Biomass. For each germplasm, the pretreatment condition that led to the greatest hemicellulose (sum of xylose and arabinose) solublization based on a multiple comparison analysis (SAS 9.1.3, Cary, NC) was selected for subsequent hydrolysis. Treatment conditions were 45 min and 1.0% acid for St6-1 leaf, 60 min and 1.5% acid for St6-3E leaf, and 60 min and 1.0% acid for St6-3F leaf. Cellulose conversion efficiencies for samples with the greatest hemicellulose solublization during acid pretreatment are summarized in Figure 1b.

In comparison to unpretreated samples with a maximum cellulose conversion of 22.82% for St6-3F leaf hydrolyzed with cellulase at 30 FPU/g of dry biomass supplemented with cellobiase, greater cellulose hydrolysis efficiency was observed for all pretreated freeze-dried samples. Greater cellulose conversion efficiency (up to 106.65%) demonstrated that cellulolytic enzymes were not inhibited by the accumulation of glucose or cellobiose. Yields over 100% may be attributed to inexact measurements during the various experimental analyses and have also been reported in other studies.[21,54] Pretreatment significantly ($p < 0.05$) improved hydrolysis efficiency of all three germplasms. Besides pretreatment, the addition of cellulase had a significant ($p < 0.05$) influence on cellulose conversion efficiency in all tests conducted, but no differences were observed between cellulase loadings of 15 and 30 FPU ($p \geq 0.05$). The addition of xylanase and enzyme interaction between cellulase activity and xylanase addition (FPU × xylanase) did not impact ($p \geq 0.05$) the extent of cellulose hydrolysis.

In comparison to hydrolyzates derived from the least lignin content pretreated biomass (Figure 1a), cellulose hydrolysis efficiencies obtained for the greatest hemicellulose solubilized samples were greater (Figure 1b), indicating that hemicellulose removal may be more effective in enhancing cellulose hydrolysis in acid-pretreated switchgrass. Plant part also impacted hydrolysis efficiency. Although slightly less in cellulose than the stem, leaf samples showed greater hemicellulose solublization during acid pretreatment and gave better cellulose hydrolysis efficiency.

Monomeric sugars were not detected in hydrolyzates of pretreated controls soaked in buffer without enzymes. One exception was 0.22% of cellulose to glucose conversion detected in the hydrolyzate of one triplicate from St6-1 leaves hydrolyzed with xylanase alone (Figure 1b). This may have been due to incomplete washing of the pretreated solids.

Xylan Conversion during Enzymatic Hydrolysis. The conversion efficiency of xylan to xylose during hydrolysis is presented in Table 5. Xylose was detected in the hydrolyzate of unpretreated switchgrass with conversion efficiency limited to 34.28%. Soaking the unpretreated biomass in citrate buffer resulted in 6.74−25.39% xylan conversion potentially because of the release of free xylose molecules from the surface of the unpretreated biomass.

Xylan conversion in least lignin content pretreated samples was not different ($p \geq 0.05$) from unpretreated samples; however, in pretreated samples with the greatest hemicellulose solublization, pretreatment had a significant ($p < 0.05$) effect on xylose yield. The addition of cellulase had a greater impact ($p < 0.05$) on xylan hydrolysis than the addition of xylanase. Damasco et al.[56] reported that, with alkali or thermal pretreatments, in which xylan removal is not as significant as with acid pretreatment, xylan hydrolysis after pretreatment was enhanced by xylanase addition. Because a large portion of hemicellulose had been removed from the switchgrass feedstock during acid pretreatment, xylanase activities reported to be 1117 units/mL (in comparison to 3760 units/mL for NS50030)[57] contained

(55) Saha, B. C.; Iten, L. B.; Cotta, M. A.; Wu, Y. V. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. *Proc. Biochem.* **2005**, *40*, 3693–3700.

(56) Damasco, M. C. T.; de Castro, A. M.; Castro, R. M.; Andrade, C.; Pereira, N. Application of xylanase from *Thermomyces lanuginosus* IOC-4145 for enzymatic hydrolysis of corncob and sugarcane bagasse. *Appl. Biochem. Biotechnol.* **2004**, *115*, 1003–1012.

(57) Garcia-Aparicio, M. P.; Ballesteros, M.; Manzanares, P.; Ballesteros, I.; Gonzalez, A.; Negro, M. J. Xylanase contribution to the efficiency of cellulose enzymatic hydrolysis of barley straw. *Appl. Biochem. Biotechnol.* **2007**, *136−140*, 353–366.

Table 5. Xylan to Xylose Conversion Efficiency (%) during Enzymatic Hydrolysis (Average Value with Standard Deviations in Parentheses)[a]

	germplasms and plant part					
hydrolysis conditions (N = 9)	St6-1 whole	St6-3E stem	St6-3F whole	St6-1 leaf	St6-3E leaf	St6-3F leaf
0 FPU, unpretreated	25.07 (1.79)	25.39 (2.91)	21.91 (1.31)	6.74 (3.18)	11.08 (0.00)	16.29 (1.77)
15 FPU, unpretreated	26.26 (1.03)	34.28 (1.90)	27.95 (1.31)	8.23 (5.19)	12.56 (1.28)	17.55 (0.00)
30 FPU, unpretreated	27.46 (2.74)	34.28 (1.90)	29.46 (0.00)	10.48 (1.30)	11.82 (1.28)	16.29 (1.77)
	least lignin content switchgrass			greatest hemicellulose solubilized switchgrass		
hydrolysis conditions (N = 18)	St6-1 whole	St6-3E stem	St6-3F whole	St6-1 leaf	St6-3E leaf	St6-3F leaf
0 FPU, pretreated	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
15 FPU, pretreated	23.97 (0.00)	39.06 (3.98)	47.23 (0.00)	52.14 (5.64)	51.95 (6.43)	74.75 (0.00)
30 FPU, pretreated	41.94 (0.00)	41.36 (0.00)	44.60 (4.54)	58.65 (0.00)	55.66 (11.13)	89.71 (0.00)
0 FPU + xylanase, pretreated	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
15 FPU + xylanase, pretreated	35.95 (0.00)	41.36 (6.89)	44.60 (4.54)	55.40 (11.29)	48.24 (6.43)	89.71 (0.00)
30 FPU + xylanase, pretreated	41.94 (0.00)	41.36 (0.00)	47.23 (0.00)	58.65 (9.78)	55.66 (0.00)	79.74 (8.63)

[a] The amount of xylose detected in the hydrolyzate was low; hence, xylose data shown here are sometimes exactly the same.

in the cellulolytic enzymes may have been sufficient to convert xylan to xylose. High xylan conversion can be achieved during hydrolysis with cellulolytic enzymes alone.[21,58] It is also believed that the performance of xylanases is better when the hemicellulose component is in a liquid rather than solid (personal communication with Novozymes). To optimize xylose yield from acid-pretreated lignocellulosic feedstocks, a mass balance calculation that accounts for compositions of both solid and filtrate collected after pretreatment is recommended.[53]

Fermentation. Hydrolyzates were fermented with a non-xylose metabolizing yeast strain to evaluate the potential of bioethanol yield from switchgrass based on cellulose (glucose) conversion.

Fermentation of Hydrolyzates from the Pretreated Least Lignin Content Biomass. Ethanol yields ranging from 0.056 to 0.083 g/g of raw biomass were obtained from hydrolyzates of least lignin content pretreated switchgrass samples (Figure 2a). The greatest ethanol yield was observed for St6-3F whole plant, which was pretreated at 121 °C/15 psi for 45 min with 1.5% acid and hydrolyzed by 30 FPU of cellulase/g of dry biomass supplemented with 0.25% (w/w) xylanase. The addition of cellulase significantly ($p < 0.05$) affected ethanol yield for all three germplasms; however, the addition of xylanase to supplement cellulolytic enzymes did not affect ethanol production ($p \geq 0.05$).

Fermentation results revealed the robustness of the yeast strain (*S. cerevisiae*, ATCC 24859) used in this study as it consumed almost all of the glucose in the hydrolyzates to produce ethanol. Although washing potentially removed some free fermentable monomeric sugars from the surface of the biomass (as explained previously), thus reducing ethanol yield to some extent, it is believed to have also removed a diverse variety of potential pretreatment-derived inhibitors, such as furfural and 5-hydroxymethylfurfural (5-HMF).[59,60] Unpretreated St6-3E stem samples, which produced 0.034 g of ethanol/g of raw biomass upon soaking in the hydrolysis buffer without any enzymes, resulted in 0.063−0.068 g of ethanol/g of raw biomass in the presence of

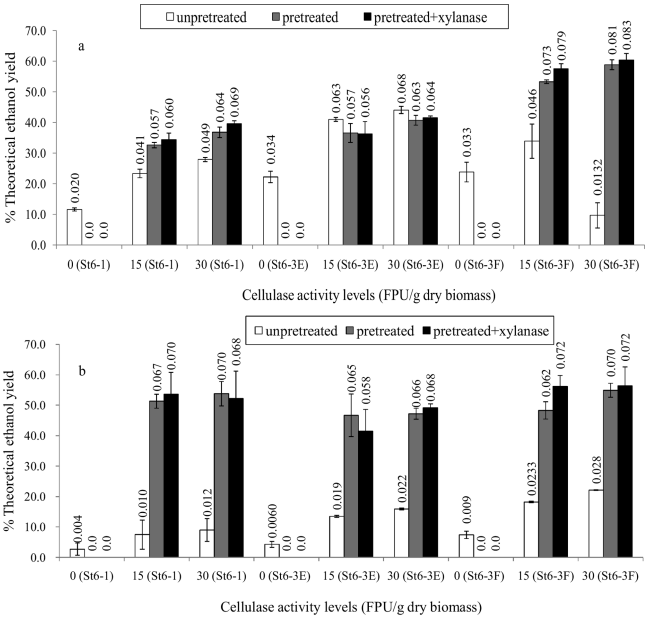

Figure 2. Ethanol yields of unpretreated and pretreated freeze-dried switchgrass plant (part) samples with the (a) least lignin content after pretreatment and (b) greatest hemicellulose solublization after pretreatment. Values on top of the columns indicate grams of ethanol per gram of unpretreated biomass.

cellulolytic enzymes (15 and 30 FPU/g of dry biomass). These yields were significantly ($p < 0.05$) greater than those from the pretreated St6-3E counterparts, and further investigation is needed to determine if the presence of inhibitors in pretreated samples could potentially be an impacting factor. Although unpretreated St6-3F samples showed greater cellulose conversion efficiency at a cellulase loading of 30 FPU/g of dry biomass than at 15 FPU/g of dry biomass (Figure 1a), fermentation results were suggestive that ethanol production was hindered at the greater cellulase level in all replicates.

Fermentation of Hydrolyzates from the Greatest Hemicellulose Solublization Biomass. Ethanol yields from hydrolyzates of freeze-dried pretreated biomass with the greatest hemicellulose solublization (all leaf samples) ranged between 0.058 and 0.072 g of ethanol/g of raw biomass and were similar among three germplasms (Figure 2b). However, for unpretreated samples, the greatest ethanol yield was limited to 0.028 g/g of raw biomass. As with the fermentation of least lignin content samples, glucose present in hydrolyzates of the

(58) Alizadeh, H.; Teymouri, F.; Gilbert, T. I.; Dale, B. E. Pretreatment of switchgrass by ammonia fiber explosion (AFEX). *Appl. Biochem. Biotechnol.* **2005**, *121*−*124*, 1133–1142.

(59) Larsson, S.; Palmqvist, E.; Hahn-Hagerdal, B.; Tengborg, C.; Stenberg, K.; Zacchi, G.; Nilvebrant, N. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzyme Microb. Technol.* **1999**, *24* (3−4), 151–159.

(60) Luo, C.; Brink, D. L.; Blanch, H. W. Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol. *Biomass Bioenergy* **2002**, *22* (2), 125–138.

greatest hemicellulose solublization was depleted during fermentation.

Pretreatment and the addition of cellulase significantly ($p < 0.05$) affected ethanol yield for all three germplasms. However, differences in ethanol yield between 15 and 30 FPU of cellulase during hydrolysis were not observed ($p \geq 0.05$). The addition of xylanase along with the cellulolytic enzymes gave no differences ($p \geq 0.05$) in the fermentation products generated. No ethanol was detected upon fermentation hydrolyzates of least lignin and greatest hemicellulose solublization samples hydrolyzed in the absence of enzymes because no glucose was released during hydrolysis (Figures 1a and 2a).

Ethanol Production Evaluation. All three switchgrass germplasms showed potential for bioethanol production with the yield being from 0.056 to 0.083 g of ethanol/g of raw biomass, with overall values being similar for the least lignin and greatest hemicellulose solublization samples. The greatest ethanol yield (0.083 g of ethanol/g of raw biomass) was obtained from St6-3F whole plant. Appropriate dilute acid pretreatment is needed for improving ethanol yield, especially when greater hemicellulose solublization is targeted during pretreatment. Recovery of sugars released into the pretreatment filtrate or pretreated biomass wash water for fermentation can further enhance the ethanol yield.

The greatest ethanol yields from acid-pretreated, freeze-dried switchgrass cultivars St6-1, St6-3E, and St6-3F were found to be 53.8, 49.3, and 60.4% of the theoretical yield, respectively. Although the optimum ethanol yield from freeze-dried switchgrass was approximately the same as that from studies on oven-dried germplasms, the maximum theoretical ethanol production efficiencies were greater for freeze-dried samples.[51] This might be due to the ability of freeze-drying technology to better preserve plant cell structure and prevent degradation of carbohydrates/sugars compared to heated drying. Chang et al.[61] reported an ethanol yield of 72% of the theoretical yield when switchgrass feedstock was first pretreated by lime and then simultaneously saccharified and fermented (SSF). Additionally, Alizadeh et al.[58] proposed that 0.2 g of ethanol/g of dry switchgrass could be obtained by SSF following pretreatment by ammonia fiber explosion (AFEX) using yeast that only consumed glucose. Apart from the loss of fermentable glucose during washing of the pretreated biomass, these differences in final ethanol yields may be attributed to variations in lignin removal and carbohydrate solublization during pretreatment as well as potential differences between SSF and separate hydrolysis and fermentation (SHF).

Conclusions

The three switchgrass germplasms investigated in this study for potential ethanol production subsequent to acid pretreatment and enzyme hydrolysis contained 24.34−30.95% glucan, 14.68−18.58% xylan, and 17.39−19.46% lignin in the whole samples. In comparison to the stem, leaves had slightly less carbohydrate content. Acid pretreatment did not result in significant delignification because of the occurrence of the acid−lignin interaction, which can lead to simultaneous lignin accumulation and degradation. However, more than 80% of the hemicellulose could be solubilized, especially in leaf samples. Perennial grasses that contain a large stem fraction are more desirable for ethanol production because stems have more cellulose and less ash.[36,62] However, in this study, which targeted greater hemicellulose removal during acid pretreatment, leaves (which typically had lower initial lignin content) were preferred because they showed greater digestibility than the stem. The optimal pretreatment conditions identified for each germplasm based on lignin removal or hemicellulose solublization were subjected to hydrolysis. Pretreatment improved cellulose conversion, and glucose produced during enzymatic hydrolysis was converted efficiently to ethanol. The greatest ethanol yield obtained was 0.083 g of ethanol/g of raw St6-3F switchgrass whole plant, equivalent to 60% of the theoretical yield. Switchgrass cultivars studied can be potential energy crops for producing bioethanol through dilute sulfuric acid pretreatment. However, further investigation of other pretreatment technologies and scale-up studies are warranted for development of an economical process.

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(61) Chang, V. S.; Kaar, W. E.; Burr, B.; Holtzapple, M. T. Simultaneous saccharification and fermentation of lime-treated biomass. *Biotechnol. Lett*. **2001**, *23*, 1327–1333.

(62) Radiotis, T.; Li, J.; Goel, K.; Eisner, R. Fiber characteristics, pulpability, and bleachability studies of switchgrass. *Proc. 1996 TAPPI Pulping Conf*. **1996**, 371–376.