DELIGNIFICATION OF SWITCHGRASS CULTIVARS FOR BIOETHANOL PRODUCTION

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Three switchgrass cultivars ('Performer', 'BoMaster', and 'Colony' switchgrass) were delignified using NaOH at varying concentrations and residence times at 121 ^{o}C for improved sugar production in enzymatic hydrolysis. Because of its greater carbohydrate/lignin ratio and the more substantial lignin reduction upon alkaline attack, 'Performer' switchgrass gave greater sugar productions under all the pretreatment conditions investigated. Maximum sugar production from 'Performer' was 425 mg/g raw biomass, which was achieved at 1% NaOH and 0.5 h. Sugar production increased with the improvement of delignification until the lignin reduction reached 30%. The more severe pretreatment conditions, which led to greater lignin reductions, did not favor the increase of sugar production because of greater solid losses. Linear models were proven effective in correlating a modified severity parameter log(M_o) to lignin reduction and sugar production of 'Performer' switchgrass.

Keywords: Delignification; Lignocellulose; Modeling; Severity; Sodium hydroxide

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INTRODUCTION

Bioethanol produced from plentiful and renewable lignocellulosic biomass is a promising alternative to fossil fuels. Switchgrass (*Panicum virgatum L.*), a warm-season perennial grass native to North America, is well adapted to the continental United States with the exception of the far Northwest. It is regarded as a potential feedstock for the production of fuel ethanol because of its excellent biomass yield, high carbohydrate content, adaptation to various soil and climate conditions, and low agricultural requirements for its production (Dien et al. 2006; Jensen et al. 2007; Keshwani and Cheng 2009). Assuming an annual biomass yield of 15 t ha^{-1}, the theoretical ethanol production from well adapted switchgrass varieties could reach 5000-6000 L ha^{-1} (Keshwani and Cheng 2009). Moreover, since conventional agricultural equipment for seeding, management, and harvesting can be used for switchgrass production, it is easy to integrate switchgrass into the existing farming operations (Lewandowski et al. 2003).

Lignocellulosic materials consist of three major components: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are structural carbohydrates that can be depolymerized through enzymatic hydrolysis to fermentable sugars for ethanol production. Lignin, on the other hand, is a complex aromatic polymer that forms a crust surrounding the carbohydrate fraction and serves as a barrier, limiting the accessibility of

carbohydrates to hydrolytic enzymes. Delignification, therefore, would improve enzymatic digestibility of lignocellulose and has been vigorously pursued in the pretreatment of lignicellulosic feedstocks. Delignification of agricultural residuals with sodium hydroxide (NaOH) has been extensively studied (MacDonald et al. 1983; Elshafei 1991; Soto et al. 1994; Sharma et al. 2002; Silverstein et al. 2007) and shows promise. MacDonald et al. (1983) reported that after a majority of the lignin in corn stover was removed (2% NaOH at 150 ^{o}C, for 15 min), 80% of the potential glucose in the raw biomass could be recovered in the subsequent hydrolysis. After investigating the pretreatment of cotton stalks using NaOH, H_2SO_4, H_2O_2, and ozone, Silverstein et al. (2007) reported that NaOH pretreatment resulted in the greatest level of delignification (65.63% at 2% NaOH, 121 ^{o}C, for 90 min) and cellulose conversion (60.8%). Switchgrass has been identified by the U.S. DOE as a preferred dedicated energy crop, and it has been demonstrated in our preliminary studies to be susceptible to alkaline pretreatment. Consequently, comprehensive investigation is warranted to improve its saccharification at reduced lignin content.

In most studies, pretreatment conditions including chemical concentration, temperature, and residence time have been evaluated as separate parameters for their effects on the subsequent enzymatic hydrolysis. However, combining these parameters into one single parameter to correlate the severity of pretreatment with its effectiveness is of interest. Overend and Chornet (1987) defined a reaction ordinate based on the combination of temperature and residence time to describe the impact of pretreatment severity on lignocellulosic components in steam-aqueous pretreatment. The reaction ordinate is defined as,

$$R_0 = t \times \exp((T_r - T_b)/14.75) \quad (1)$$

where t is the residence time (min), T_r is the treatment temperature (^{o}C), T_b is the base temperature (100 ^{o}C), and 14.75 is the activation energy based on the assumption that the reaction is hydrolytic and the overall conversion is first order. The logarithm of the reaction ordinate (log R_o) is defined as the severity of the pretreatment. Chum et al. (1990) incorporated the effect of hydrogen ion concentration and proposed a modified severity factor for acid-catalyzed treatments:

$$R_0' = [H^+] \times t \times \exp((T_r - T_b)/14.75) \quad (2)$$

This modified severity factor has seen successful application in dilute-acid pretreatment (Kabel et al. 2007). Silverstein et al. (2007) made a further modification to the severity factor for alkaline pretreatment,

$$M_0 = C^n \times t \times \exp((T_r - T_b)/14.75) \quad (3)$$

where C is the chemical concentration (wt %), and n is an arbitrary constant. This factor has been used to describe the linear relationship between pretreatment severity and lignin reduction in alkaline pretreatment of cotton stalks (Silverstein et al. 2007). However, the

predictive ability of the model was not assessed due to insufficient data. Since developing models that could predict the pretreatment results based on one single severity parameter not only enables an easy comparison of outcomes, but also could considerably facilitate the process control, further studies are required to evaluate the effectiveness of using this modified severity factor to predict the alkaline delignification of biomass.

In this study, three lowland switchgrass cultivars ('Performer', 'BoMaster', and 'Colony' switchgrass) were delignified using different combinations of NaOH concentration and residence time to determine the best cultivar for sugar production. Also examined were the correlation between delignification efficiency and sugar production from enzymatic hydrolysis of the biomass, as well as the development of a regression model to predict delignification based on the modified severity factor. Considerations were also given to the relationship between pretreatment severity and sugar production.

EXPERIMENTAL

Biomass Preparation

'Performer', 'BoMaster', and 'Colony' switchgrass, three cultivars originally developed as animal feed (Burns et al. 2008a, 2008b, 2010), were used as feedstocks in this research. Switchgrass biomass was obtained from Central Crops Research Station near Clayton, North Carolina. For each cultivar, a harvest strip was taken randomly from each quarter of the field, and four strips were combined to form one bulk sample, which was oven dried at 50 ^{o}C for 72 hours. The dried plants were ground to pass through a Wiley mill fitted with a 2 mm screen. The prepared biomass was collected in plastic bags, sealed, and delivered to the Bio-products Development Lab in the Department of Biological & Agricultural Engineering at NCSU. The biomass was stored at room temperature during the course of the study, and its chemical composition was analyzed before pretreatment.

Delignification

To determine the best switchgrass cultivars for sugar production, biomass feedstocks were subjected to NaOH delignification at 121 ^{o}C in an autoclave. Pretreatments at different combinations of NaOH concentration (0.5, 1.0, 2.0 and 3.0%, w/v) and residence time (0.25, 0.5, 1.0, 1.5 h) were carried out. Delignifications at lower temperatures and extended residence times were also conducted to increase the size of data set for model development. Four g of biomass sample and 40 mL NaOH solution of the desired concentration were mixed in a serum bottle, forming a slurry of 0.1 g/mL. All serum bottles were sealed and crimped before pretreatment. The pretreated biomass was recovered by filtration and washed with 400 mL of deionized water to remove excess alkali and dissolved byproducts that might inhibit enzymes in the subsequent hydrolysis. About 1 g of the pretreated biomass was dried at 105 ^{o}C for 24 h to determine the moisture content, and the rest was stored in a sealed plastic bag at 4 ^{o}C for enzymatic hydrolysis. The percentage lignin reduction of biomass was calculated as follows,

$$\%\ Lignin\ reduction = \frac{\%LR - \%LP}{\%LR} \times 100 \quad (4)$$

where LR is the lignin content of raw biomass and LP is the lignin content of pretreated biomass.

Enzymatic Hydrolysis

The digestibility improvement of switchgrass was determined by total reducing sugar yield of pretreated biomass in enzymatic hydrolysis. Wet pretreated biomass (1 g, dry basis) was immersed in 30 mL of 50 mM sodium citrate buffer (pH 4.8) in a 250 mL Erlenmeyer flask. Cellulase (NS-50013) from *Trichoderma reesei* (E.C. 3.2.1.4) was added, supplemented with cellobiase (NS-50010) from *Aspergillus niger* (E.C. 3.2.1.21) to prevent cellobiose inhibition. To eliminate the impact of enzyme limitation on sugar yield, excessive cellulase of 35 FPU (filter paper unit)/g dry biomass and cellobiase of 61.5 CBU (cellobiase unit)/g dry biomass were added. FPU is defined as the amount of enzyme that produces 1 μmol of glucose from filter paper per minute, and CBU is defined as the amount of enzyme that produces 2 μmol of glucose from cellobiose per minute. Enzymes were obtained from Novozymes North America, Inc. (Franklinton, North Carolina, USA). The activities of cellulase and cellobiase were 80 FPU/mL and 277 CBU/mL, respectively. Sodium azide (0.3%, w/v) was added into the mixture to mitigate microbial contamination during hydrolysis. The flasks were incubated at 55 ^{o}C, and shaken at 150 rpm in an air bath shaker for 72 h. After the hydrolysis time elapsed, the flasks were immediately chilled in an ice bath to avoid further reaction. Hydrolysate was collected by centrifugation at 10000 rpm for 5 min. The supernatant was stored at minus 80 ^{o}C for sugar analysis.

Analytical Methods

Total solids, ash, structural carbohydrates, and lignin of raw and pretreated biomass were determined using Laboratory Analytical Procedures (LAP) established by National Renewable Energy Laboratory (NREL) (Sluiter et al. 2005a,b, 2008). Total reducing sugars in hydrolysate were measured using the 3,5-dinitrosalicylic acid method adapted from Miller (1959) and Ghose (1987). The carbohydrates in raw biomass were determined by measuring cellulose and hemicellulose derived monosaccharides (glucose, xylose, galactose, arabinose, and mannose) using high performance liquid chromatography (HPLC). The HPLC system was equipped with a Bio-Rad Aminex HPX-87P column (300mm×7.8mm) tailored for the analysis of lignocellulose-derived sugars, a Bio-Rad Micro-Guard column, a thermostatted autosampler, a quaternary pump, and a refractive index detector. The standards used were glucose, xylose, galactose, arabinose, and mannose at concentrations of 0.5, 2.0, 5.0, 7.5, 10.0 g/L. The analytical column was operated at 80 °C with HPLC grade water as the mobile phase at a flow rate of 0.6 mL/min. The samples were injected at 10 μL and the acquisition time was 35 min. A post-run time of 25 min was included between injections to allow for late-eluting compounds to come off the column.

Data Analysis

All treatments in this study were conducted in triplicate. The GLM procedure in SAS 9.1 software (SAS Institute Inc., Cary, NC) was used for all data analysis. Analysis of variance (ANOVA) was used to determine the effects of various factors on pretreatments. Tukey simultaneous tests were conducted to determine the statistical differences between treatments. A 95% confidence level was applied for all analysis.

Modeling

Models correlating lignin reduction during pretreatment and sugar production during enzymatic hydrolysis with pretreatment severity were developed based on the best swichgrass cultivar and a modified severity factor of $M_0 = t \times C^n \times \exp((T_r - T_b)/14.75)$. A training data set, which included 67 data points for model development, was obtained by conducting pretreatments using 0.5 to 3% NaOH at 121 ^oC for 0.25 to 1.5 h, 50 ^oC for 1 to 168 h, and 21 ^oC for 1 to 336 h. To evaluate the predictive ability of the models, a testing data set, which included 12 data points, was obtained by conducting another batch of pretreatment using 0.75% NaOH at 121 ^oC for 0.25 to 1.5 h, 50 ^oC for 1 to 12 h, and 21 ^oC for 3 to 24 h. The models were validated by plotting actual data values against model predicted values. Linear regression models were fit to the predicted and actual values to obtain the intercept, slope, and R^2 values, which were used to evaluate the predictive ability of the models.

RESULTS AND DISCUSSION

Characterization of Switchgrass Cultivars

The key components of the biomass samples from the three switch-grass cultivars were analyzed (Table 1). It was found that the carbohydrate fraction of switchgrass feedstock was 48.3 to 53.5% of the total biomass. Glucan, which is basically cellulose derived from plant cell wall, and xylan, which is the primary building block of hemicellulose, represented the major carbohydrates in the feedstocks. Galactan and arabinan are the two minor hemicellulose constituents and accounted for only a small fraction of the biomass composition, while mannan was not detected. Lignin, the major target of alkaline attack, constituted 21.4 to 23.0% of the total biomass. Other undefined components were mainly nonstructural compounds including protein, waxes, fats, resins, gums, chlorophyll, etc. (Kuhad and Singh 1993; Sluiter et al. 2005c). Due to its greater carbohydrate/lignin ratio,'Performer'switchgrass seemed more promising than the other two cultivars for sugar production. This agrees with the fact that 'Performer' is a hybrid cultivar developed as an animal feed with greater dry matter digestibility, while 'BoMaster' and 'Colony' were intended for greater dry biomass yield.

Delignification of Switchgrass Cultivars

NaOH pretreatment was effective in removing lignin from various switchgrass cultivars. Delignification efficiency differed ($P<0.05$) for NaOH concentration, residence time and among cultivars (Fig. 1). The maximum lignin reductions for 'Performer', 'BoMaster', and 'Colony' switchgrass, respectively, reached 73.6, 68.5, and 67.9%.

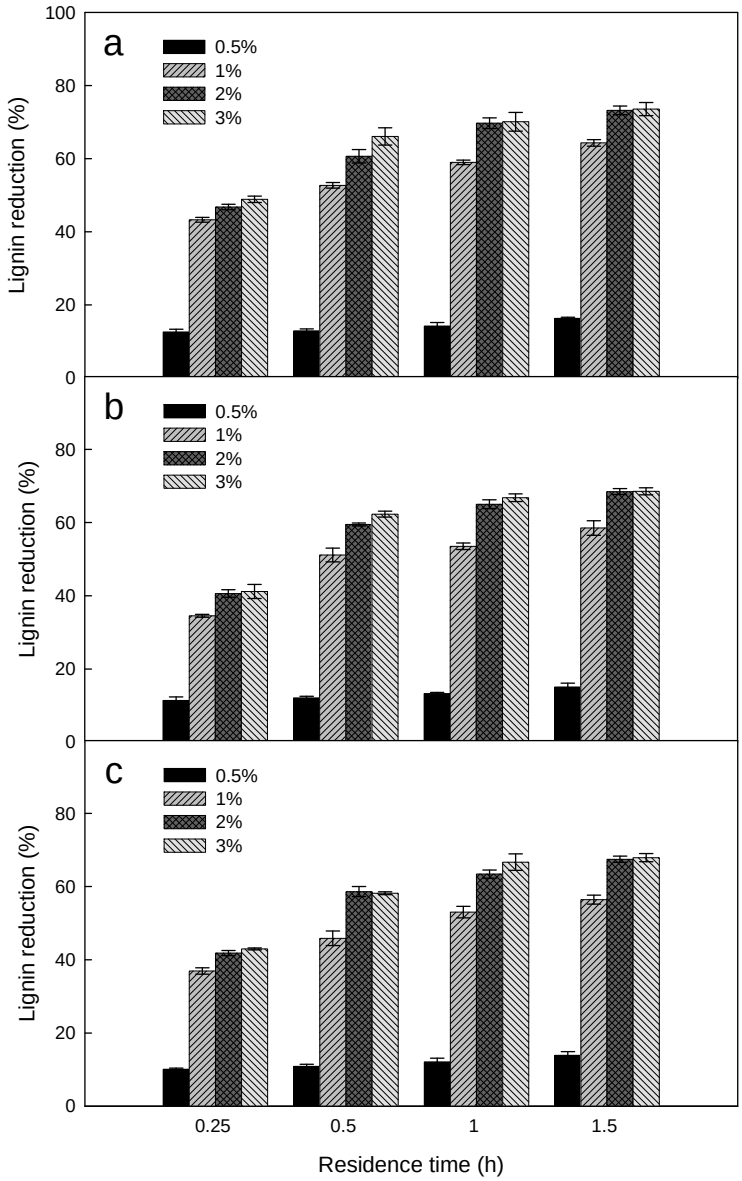

Figure 1. Lignin reductions of (a) 'Performer', (b) 'BoMaster', and (c) 'Colony' switchgrass at different combinations of NaOH concentration and residence time at 121 ^{o}C

These reductions were all recorded at the greatest NaOH concentration for the longest residence time, indicating a close relationship between lignin reduction and pretreatment severity.

Based on the lignin reduction results, 'Performer', the cultivar with the least lignin content, was most susceptible to NaOH delignification, indicating the greatest potential for sugar production from subsequent enzymatic hydrolysis. Better delignification effectiveness, when associated with improved pretreatment severity, normally indicates greater digestibility of pretreated biomass. However, since increasing

pretreatment severity inevitably leads to greater biomass solubilization, lignin reduction may not be an accurate indicator for overall pretreatment efficiency.

Table 1. Chemical Composition of 'Performer', 'BoMaster', and 'Colony' Switchgrass

Component	Dry weight (%)		
	Performer	BoMaster	Colony
Glucan	32.0	29.9	28.2
Xylan	17.9	16.6	16.3
Galactan	1.73	1.68	1.82
Arabinan	1.87	1.94	2.02
Lignin*	21.4	22.9	23.0
Ash	3.77	4.07	3.94
Other	21.3	22.9	24.7
Carbohydrate/lignin	2.50	2.19	2.10
* Including acid soluble lignin and acid insoluble lignin			

Sugar Production of Switchgrass Cultivars

Unlike lignin reduction, the production of total reducing sugars in enzymatic hydrolysis was not substantially affected by NaOH concentration and residence time (Fig. 2). Increasing pretreatment severity, although favored the improvement of biomass digestibility, resulted in more intense biomass solubilization, which reduced the sugar production potential of pretreated biomass. For example, the sugar production from 'Performer' switchgrass pretreated using 3% NaOH for 1.5 h was 31.4% higher than that using 1% NaOH for 0.25 h. However, due to the higher solid loss at greater pretreatment severity (57.0% at 3% NaOH, 1.5 h, and 37.6% at 1% NaOH, 0.25 h), there was not significant difference between the two pretreatments in terms of total reducing sugar yield based on raw biomass. 'Performer' switchgrass gave the best performance in sugar production, which is in accordance with the composition analysis of the raw biomass and lignin reduction results. The maximum sugar productions of 'Performer', 'BoMaster', and 'Colony' switchgrass were 425, 386, and 380 mg/g raw biomass, which were, respectively, achieved at 1% NaOH and 0.5 h, 2% NaOH and 1 h, and 2% NaOH and 1.5 h. The elevated carbohydrate content and its great susceptibility to alkaline delignification make 'Performer' switchgrass a promising feedstock for bioethanol production.

Correlation between Lignin Reduction and Sugar Production

There is a strong consensus that lignin removal is essential to the improvement of biomass digestibility, and much work has been done to explore this relationship (Jung and Vogel 1986; Sewalt et al. 1997; Zhu et al. 2008; Xu et al. 2010). The correlation between lignin reduction and raw biomass-based sugar production, however, has not been fully investigated. Total sugar production based on raw biomass is normally the ultimate criterion to evaluate pretreatment performance. An improvement in digestibility, however, does not necessarily equate to a greater total sugar production. Consequently, it is important to study the effect of delignification on raw biomass-based sugar production to better understand the effectiveness of pretreatment.

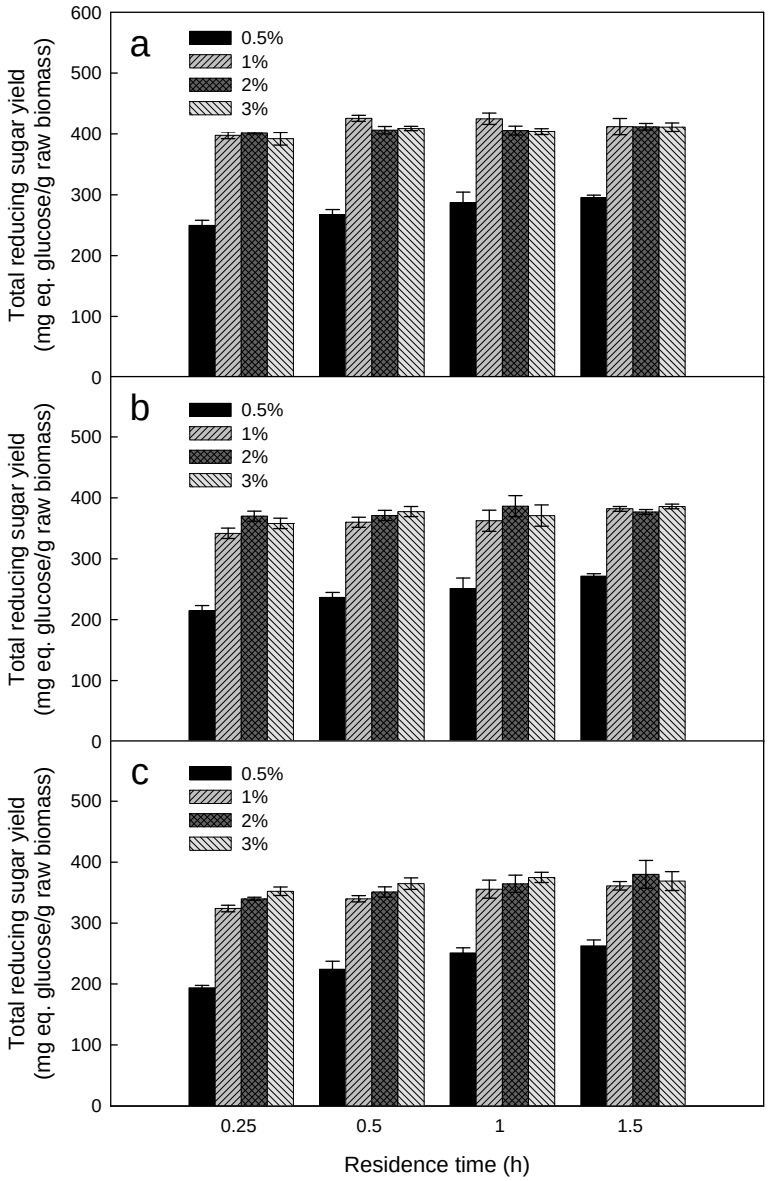

Figure 2. Sugar productions of (a) 'Performer', (b) 'BoMaster', and (c) 'Colony' switchgrass pretreated at different combinations of NaOH concentration and residence time at 121 ^{o}C

'Performer' switchgrass was subjected to NaOH delignification at two lower temperatures (50 and 21 ^{o}C) to obtain a more robust data set. The results showed that the reducing sugar yield of pretreated biomass increased with the improvement of delignification, and an exponential curve gave the best fit to the data points (Fig. 3a). The maximum sugar yield was 915 mg/g pretreated biomass, which was obtained at the maximum lignin reduction (74.6%). However, solid losses increased linearly with the delignification improvement because the more severe pretreatment conditions resulted in more intensive biomass solubilizaiton (Fig. 3b). This could substantially reduce the total

carbohydrate available for sugar production in the subsequent enzymatic hydrolysis. As a result, the total reducing sugar yield based on raw biomass leveled off after the lignin reduction reached approximately 30% (Fig. 3c). Below 30% lignin reduction, sugar production showed a positive linear correlation to the lignin reduction level. Above 30% lignin reduction, sugar production plateaued and averaged 410 mg/g raw biomass. Hence pretreatment severities resulted in greater than 30% lignin reduction are not recommended because solid losses limit total sugar production.

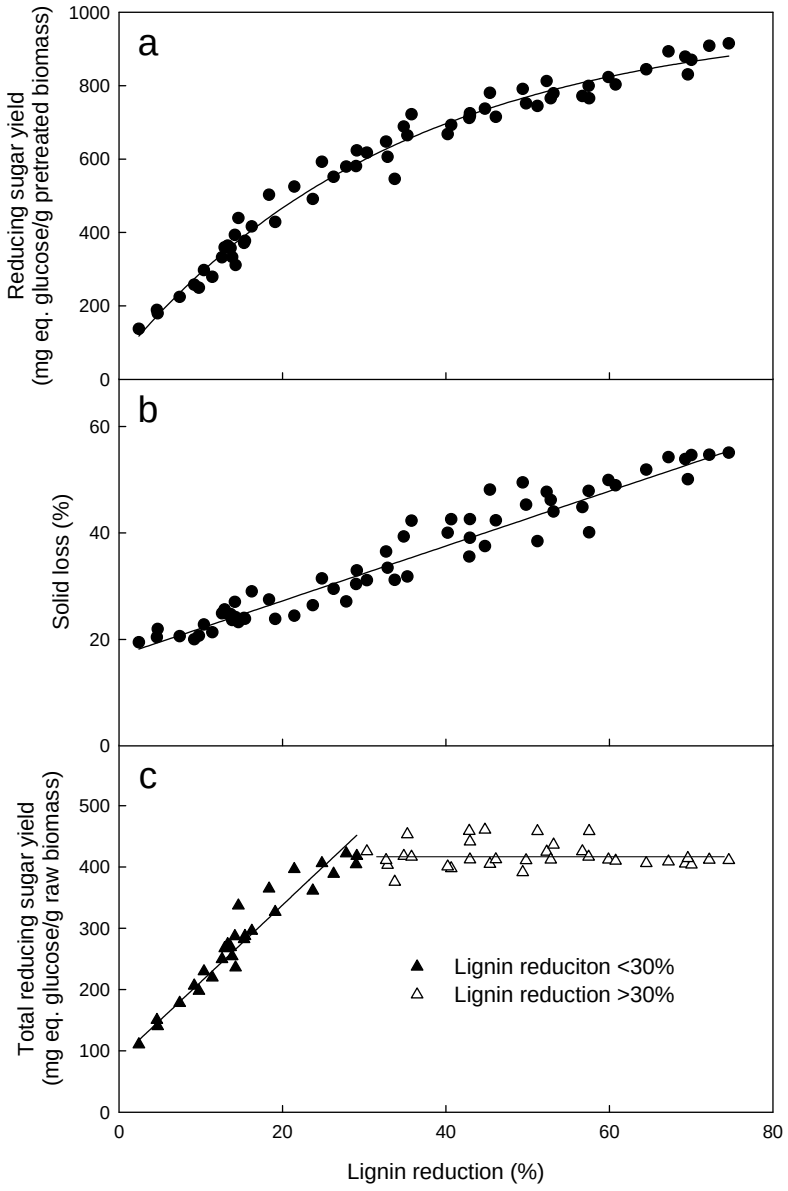

Figure 3. Correlation between lignin reduction of 'Performer' switchgrass and (a) sugar production of pretreated biomass, (b) solid loss, and (c) total sugar production based on raw biomass

Modeling

A linear model correlating a modified pretreatment severity parameter that combines the effects of time, temperature and NaOH concentration with lignin reduction was established according to Silverstein et al (2007). The model equation is as follows:

$$\%\ Lignin\ reduction = a \times \log M_0 + b = a \times \log(t \times C^n \times \exp((T_r - T_b)/14.75)) + b \quad (5)$$

The model was developed based on a training data set, with *a*, *b*, and *n* determined using nonlinear least squares regression software (CurveFitter 4.5.4, Institute of Mathematics and Statistics). Applying the parameters estimated, the statistical linear regression model is as follows:

$$\%\ Lignin\ reduction = 12.977 \times \log(t \times C^{4.322} \times \exp((T_r - T_b)/14.75)) + 35.688$$
$$(R^2 = 0.879) \quad (6)$$

The model was validated by applying it to a testing data set. Figure 4 shows the correlation between predicted and actual values for the linear model. The R^2 of 0.979 with an interception of -2.268 and slope of 1.075 indicates good predictive ability of the model.

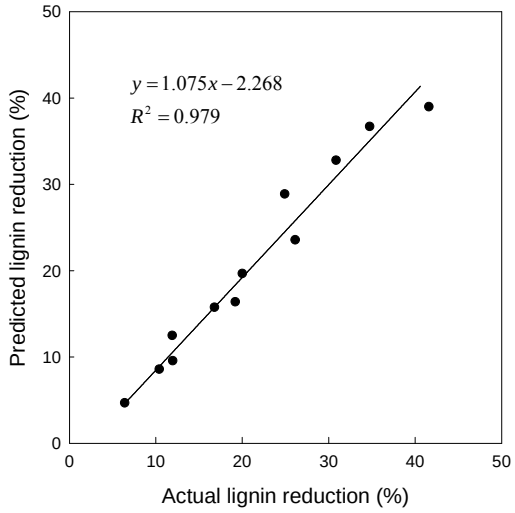

Figure 4. Linear regression model presenting fit between predicted and actual values for lignin reduction of 'Performer' switchgrass

Since total sugar production of the raw biomass and delignification efficiency are linearly related when lignin reductions are less than 30%, the usage of a linear model to correlate raw biomass-based sugar production with pretreatment severity should also be appropriate. The model equation is as follows:

$$Sugar\ production\ (mg/g\ raw\ biomass) =$$
$$a \times \log(t \times C^n \times \exp((T_r - T_b)/14.75)) + b \quad (7)$$

The model was developed based on a subset of training data that included conditions that resulted in lignin reductions of less than 30%. Applying the parameters estimated, the statistical linear regression model is as follows:

$$Sugar\ \ production\ (mg/g\ \ raw\ biomass) = 71.723 \times \log(t \times C^{6.556} \times \exp((T_r - T_b)/14.75)) + 396.746 \quad (R^2 = 0.872) \qquad (8)$$

Therefore, to predict the raw biomass-based sugar production of 'Performer' switchgrass, the lignin reduction should be first predicted using equation (6). If the calculated lignin reduction is lower than 30%, equation (8) can be applied for the prediction of sugar production. Otherwise, 410 mg/g raw biomass can be a reasonable prediction. The model was validated by applying it to a testing data set. Figure 5 shows the correlation between predicted and actual values for the linear model when the predicted lignin reductions are less than 30%. The R^2 of 0.874 was substantially lower than that of delignification model, which was probably due to the reduced amount of testing data used for model validation (the testing data set was divided into two subsets to validate the two respective parts of the sugar production model). The interception and slope of 5.794 and 0.937, respectively, indicated reasonable predictive ability of the model. At the pretreatment severities resulting in lignin reductions greater than 30%, the average total sugar production was 382 mg/g raw biomass, which is 6.85% lower than the predicted value of 410 mg/g raw biomass. This difference can also be attributed to the effect of the reduced size of the testing data set. Overall, the total sugar production of 'Performer' switchgrass can be effectively predicted using a modified severity parameter based on temperature, alkaline concentration, and residence time.

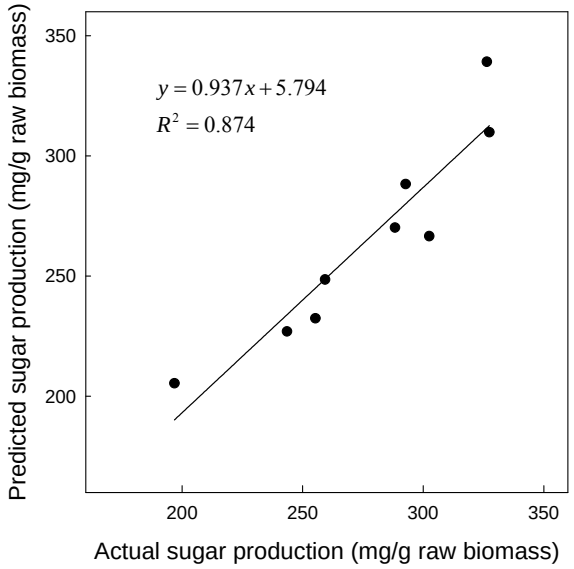

Figure 5. Linear regression model presenting fit between predicted and actual values for sugar production of 'Performer' switchgrass at lignin reductions less than 30%

CONCLUSIONS

1. Among the three switchgrass cultivars studied, 'Performer' switchgrass is a more promising feedstock for bioethanol production due to its high carbohydrate content and great susceptibility to alkaline delignification.
2. Although severe pretreatment conditions normally lead to greater lignin reductions, which is essential for digestibility improvement of biomass, the greater solid losses induced compromises in the total sugar production by enzymatic hydrolysis. Lignin reductions greater than 30% didn't favor the improvement of total sugar production from 'Performer' switchgrass.
3. A modified severity parameter that combines the effects of residence time, alkaline concentration, and temperature can be used to effectively predict the lignin reduction and sugar production of 'Performer' switchgrass.

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REFERENCES CITED

Burns, J. C., Godshalk, E. B., and Timothy, D. H. (2008a). "Registration of 'Performer' switchgrass," *J. Plant Registrations* 2(1), 31-32.

Burns, J.C., Godshalk, E. B., and Timothy, D. H. (2008b). "Registration of 'BoMaster' switchgrass," *J. Plant Registrations* 2(1), 29-30.

Burns, J. C., Godshalk, E. B., and Timothy, D. H. (2010). "Registration of 'Colony' lowland switchgrass," *J. Plant Registrations* 4: (in press).

Chum, H. L., Johnson, D. K., Black, S. K., and Overend, R. P. (1990). "Pretreatment-catalyst effects and the combined severity parameter," *Appl.Biochem. Biotechnol.* 24-25, 1-14.

Dien, B. S., Jung, H. G., Vogel, K. P., Casler, M. D., Lamb, J. F. S., Iten, L., Mitchell, R. B., and Sarath, G. (2006). "Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass," *Biomass Bioenergy* 30, 880-891.

Elshafei, A. M. (1991). "The saccharification of corn stover by cellulase from *Penicillium funiculosum*," *Bioresour. Technol.* 35, 73-80.

Ghose, T. K. (1987). "Measurement of cellulase activities," *Pure Appl. Chem.* 59(2), 257-268.

Jensen, K., Clark, C. D., Ellis, P., English, B., Menard, J., Walsh, M., and de la Torre Ugarte, D. (2007). "Farmer willingness to grow switchgrass for energy production," *Biomass Bioenergy* 31, 773-781.

Jung, H. G., and Vogel, K. P. (1986). "Influence of lignin on digestibility forage cell wall material," *J. Anim. Sci.* 62, 1703-1712.

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

Keshwani, D. R., and Cheng, J. J. (2009). "Swithgrass for bioethanol and other value-added applications: A review," *Bioresour. Technol.* 100, 1515-1523.

Kuhad, R. C., and Singh, A. (1993). "Lignocellulose biotechnology: Current and future prospects," *Crit. Rev. Biotechnol.* 13, 151-172.

Lewandowski, I., Scurlock, J. M. O., Lindvall, E., and Christou, M. (2003). "The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe," *Biomass Bioenergy* 25, 335-361.

MacDonald, D. G., Bakhshi, N. N., Mathews, J. F., and Roychowdhury, A. (1983). "Alkali treatment of corn stover to improve sugar production by enzymatic hydrolysis," *Biotechnol. Bioeng.* 25, 2067-2076.

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Anal. Chem.* 31, 426-428.

Overend, R. P., and Chornet, E. (1987). "Fractionation of lignocellulosics by steam-aqueous pretreatments," *Philos. Trans. R. Soc. Lond.* 321, 523-536.

Sewalt, V. J. H., Ni, W., Jung, H. G., and Dixon, R. A. (1997). "Lignin impact on fiber: Increased enzymatic digestibility of genetically engineered tobacco (*Nicotiana tabacum*) stems reduced in lignin content," *J. Agric. Food Chem.* 45, 1977-1983.

Sharma, S. K., Kalar, K. L., and Grewal, H. S. (2002). "Enzymatic saccharification of pretreated sunflower stalks," *Biomass Bioenergy* 23, 237-243.

Silverstein, R. A., Chen, Y., Sharma-Shivappa, R. R., Boyette, M. D., and Osborne, J. (2007). "A comparison of chemical pretreatment methods for improving saccharification of cotton stalks," *Bioresour. Technol.* 98, 3000-3011.

Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Wolfe, J. (2005a). "Determination of total solids in biomass and total dissolved solids in liquid process samples," Laboratory Analytical Procedure (LAP). NREL/TP-510-42621. National Renewable Energy Laboratory, Golden, Colorado.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., and Templeton, D. (2005b). "Determination of ash in biomass," Laboratory Analytical Procedure (LAP). NREL/TP-510-42622. National Renewable Energy Laboratory, Golden, Colorado.

Sluiter, A., R. Ruiz, C. Scarlata, J. Sluiter, and Templeton, D. (2005c). "Determination of extractives in biomass," Laboratory Analytical Procedure (LAP). NREL/TP-510-42619. National Renewable Energy Laboratory, Golden, Colorado.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D., (2008). "Determination of structural carbohydrates and lignin in biomass," Laboratory Analytical Procedure (LAP). NREL/TP-510-42618. National Renewable Energy Laboratory, Golden, Colorado.

Soto, M. L., Dominguez, H., Nunez, M. J., and Lema, J. M. (1994). "Enzymatic saccharification of alkali-treated sunflower hulls," *Bioresour. Technol.* 49, 53-59.

Xu, J., Cheng, J. J., Sharma-Shivappa, R. R., and Burns, J. C. (2010). "Sodium hydroxide pretreatment of switchgrass for ethanol production," *Energy Fuels* 24(3), 2113-2119.

Zhu, L., O'Dwyer, J. P., Chang, V. S., Granda, C. B., and Holtzapple, M. T. (2008). "Structural features affecting biomass enzymatic digestibility," *Bioresour. Technol.* 99, 3817-3828.

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