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# Laboratory scale optimization of alkali pretreatment for improving enzymatic hydrolysis of sweet sorghum bagasse

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## Abstract

Sweet sorghum has been identified as a promising feedstock for biological conversion to fuels as well as other chemicals. The lignocellulosic stalk of sweet sorghum, called sweet sorghum bagasse (SSB) is a potential source of lignocellulosic biofuel. The primary goal of this study was to determine optimal alkali (lime : Ca(OH)<sub>2</sub> and lye : NaOH) pretreatment conditions to obtain higher yield of total reducing sugar while reducing the lignin content for biofuel production from SSB. Biomass conversion and lignin removal were simultaneously optimized through four quadratic models analyzed by response surface methodology (RSM). The optimal conditions for lime pretreatment was 1.7% (w/v) lime concentration, 6.0% (w/v) SSB loading, 2.4 h pretreatment time with predicted yields of 85.6 total biomass conversion and 35.5% lignin reduction. For lye pretreatment, 2% (w/v) alkali, 6.8% SSB loading and 2.3 h duration were the optimal levels with predicted biomass conversion and lignin reduction of 92.9% and 50.0%, respectively. More intensive pretreatment conditions removed higher amounts of hemicelluloses and cellulose. Fourier transform infrared spectroscopy (FTIR) spectrum and scanning electron microscope (SEM) image revealed compositional and microstructural changes caused by the alkali pretreatment.

**Keywords:** lignocellulosic biomass; sweet sorghum bagasse; alkali pretreatment; enzymatic hydrolysis; response surface methodology.

## Symbols and abbreviations

SSB	Sweet sorghum bagasse
RSM	Response surface methodology
TRS	Total reducing sugar
NaOH	Lye; Sodium hydroxide
Ca(OH) <sub>2</sub>	Calcium hydroxide or lime
FTIR	Fourier transform infrared spectroscopy
SEM	Scanning electron microscope
% w/v	Percentage weight per volume
h	Hours
CCD	Central composite design
KBr	Potassium bromide
$x_1$	Alkali concentration % w/v

$x_2$	SSB loading % w/v
$x_3$	Reaction time (h)
$Y_{11}$	Biomass conversion % by lime pretreatment
$Y_{21}$	Lignin reduction % by lime pretreatment
$Y_{12}$	Biomass conversion % by sodium hydroxide pretreatment
$Y_{22}$	Lignin reduction % by sodium hydroxide pretreatment
C	Cellulose content of SSB in %
H	Hemicellulose content of SSB in %
Lig	Lignin content of SSB
Lpt	Lignin content of pretreated SSB

## 1. Introduction

Sweet sorghum (*Sorghum bicolor* (L.) Moench) has been considered an attractive raw material for the production of biofuel, owing to its greater biomass yield per hectare, high photosynthetic efficiency, and wide adaptability to harsh environmental conditions (Rubin, 2008). Previous studies found that sweet sorghum contains 43.6–58.2% soluble sugars in the stalk (Amaducci, Monti, Venturi, 2004; Antonopoulou, Gavala, Skiadas, Angelopoulos, Lyberatos, 2008), 22.6–47.8% insoluble cellulose and hemicelluloses (Antonopoulou *et al.*, 2008) and 14.1–20.8% lignin (Li, Balan, Yuan, Dale, 2009). Solid state fermentation of stem (Mamma *et al.*, 1996; Yu, Zhang, Tan, 2008); and sorghum juice fermented to ethanol are two common approaches of bioethanol production from sweet sorghum. However, large scale sweet sorghum juice extraction results in a large amount of biomass waste referred to as sweet sorghum bagasse (SSB). Considering SSB's high content of cellulose and hemicellulose, it has been investigated for fuel-ethanol production through: pretreatment, enzymatic hydrolysis, and yeast fermentation (Ban, Yu, Zhang, & Tan, 2008; Li *et al.*, 2009; Mehmood *et al.*, 2009; Sipos *et al.*, 2009). As SSB has been identified as an attractive feedstock, several pretreatment and conversion methods have been investigated, such as ammonia fiber explosion (Li *et al.*, 2009), steam (Sipos *et al.*, 2009), liquid hot water (Dogaris, Karapati, Mamma, Kalogeris, Kekos, 2009), lime (Liang, Tang, Siddaramu, Choudhary, Umagiliyage, 2012a; Liang *et al.*, 2012b), and microwave pretreatment (Choudhary *et al.*, 2012). However, none of the published literature reported response surface optimization of lime and lye pretreatment of sweet sorghum bagasse.

Alkali pretreatment such as exposure to lime is an economical pretreatment process that uses a suspension of ground biomass in lime, at relatively low temperatures and pressures compared with other methods (Beukes, Brett, Pletschke, 2010). It is an attractive pretreatment technology, since it has low formation of fermentation inhibitors; increases pH and accessibility of the surface exposed to enzymatic hydrolysis through the removal of acetyl and uronic acid substituents on hemicelluloses and is a low cost alternative for lignin solubilization (Beukes *et al.*, 2010; Chang & Holtzapple, 2000). Though it has a relatively slower mode of action than other pretreatment methods and solubilizes a small percentage of the hemicelluloses, low cost and safe handling make lime pretreatment attractive (Rabelo, Filho, Costa, 2009). Lime pretreatment research has led to high conversion of different lignocellulosic feedstocks, such as sugarcane bagasse (Beukes *et al.*, 2010; Rabelo *et al.*, 2009), switch grass (Chang, Burr, Holtzapple, 1997), rice straw (Park *et al.*, 2010) and wheat straw (Saha & Cotta, 2007). However, we did not find any

literature reporting studies on response surface methodology for optimizing lime pretreatment for enzymatic saccharification of SSB.

Chemical pretreatment with lye (sodium hydroxide :NaOH) may eliminate or modify lignin by breaking bonds and loosening cross links between xylan and lignin, so resulting higher porosity (Mirahmadi, Kabir, Jeihanipour, Karimi, Taherzadeh, 2010). The efficiency of lye pretreatment depends on NaOH concentration, treatment time, temperature, and inherent characteristics of the biomass used (McIntosh & Vancove, 2010; Mirahmadi *et al.*, 2010).

In our prior studies on lime pretreatment of SSB (Umagiliyage, Choudhary, Liang, Siddaramau, Haddock, 2010; Liang *et al.*, 2012b), four lime doses (g per g of SSB): 0.05, 0.1, 0.15, and 0.2; two water contents (ml per g of SSB): 10 and 20; and three treatment durations: 1, 2 and 3 h were used, where higher losses of lignin and xylan occurred at higher lime doses. However those studies did not include process optimization by response surface methodology to recommend best alkali pretreatment conditions for enzymatic hydrolysis of SSB.

In this article we are reporting optimization of process parameters for alkali pretreatment of sweet sorghum bagasse by response surface methodology for maximum removal of lignin as well as maximum release of fermentable sugar by enzymatic saccharification ultimately leading to increased yield of fuel ethanol.

## **2. Materials and methods**

### **2.1. Feedstock preparation and composition analysis**

SSB was obtained from a commercial farm in southern Illinois region, just after juice was expressed from stripped sweet sorghum stalks. Then SSB was washed with distilled water and dehydrated under the sun. Dried SSB was ground using Wiley Model 4 mill (Arthur H. Thomas Co., Phil, PA, USA) containing a 2 mm outlet mesh. The ground biomass screened to a size range of 1.0 to 0.5 mm (US mesh 18 & 35) were used in this study in order to use a standard particle size range recommended by the NREL protocol for composition analysis (Sluiter *et al.*, 2004). Structural polysaccharides and lignin in SSB were quantified by following National Renewable Energy Laboratory (NREL) protocols as described in Sluiter *et al.*, 2004. Standard procedures were used for measure the total solids (NREL, 2008) and ash (NREL, 2005) in biomass. The ground biomass was secured in closed containers under refrigeration until used for pretreatment.

### **2.2. Pretreatment of SSB**

The pretreatment of SSB was done using different quantities (% w/v) of sodium hydroxide and calcium hydroxide solutions separately. In all cases, 15 ml polypropylene conical centrifuge tubes (BD Falcon, CA, USA) were utilized as pretreatment apparatuses. 10 ml of alkali solution of desired dose was mixed with the amount of SSB in each reactor according to experimental design, followed by capping tightly, and placing in boiling water for 10 min for rapidly rising to 100 °C. The reactor tubes were then mounted randomly on a rotating drum (75 rpm) in preheated oven at 100 °C (Figure 1).

Then freeze drying (VirTis Bench top 2k, SP industries Inc. Gardiner, NY) was performed to pretreated biomass. The freeze dried samples in reactor tubes were capped and stored in a desiccator at 4 °C. Samples were removed as needed for compositional analysis, SEM imaging, FTIR analysis, and enzymatic hydrolysis.

### **2.3. Enzymatic hydrolysis**

The procedure of Liang *et al* (2012 a) was followed for enzymatic hydrolysis of pretreated SSB. From each pretreated sample, 0.2 g (freeze dried) was weighed to a 125 ml flask. Sodium azide (0.7 ml, 0.01 g/ml) and 1 ml of 1.0 M citrate buffer (pH 4.8) were added to the flask and then final volume was brought to 20 ml by adding distilled water. Two commercially available enzymes from Genencor (Rochester, NY, USA); Accellerase<sup>®</sup> 1500 (cellulases) at 0.24 mL/g of SSB and Accellerase<sup>®</sup> XC (xylanase) at 0.25 ml/g of SSB were added to each flask. followed by shaking incubation at 50 °C and 100 rpm (Innova 4230, New Brunswick scientific, Edison, NJ). At 0, 12, 24, and 48 h time intervals, 1.0 ml samples from each flask were centrifuged and analyzed for total reducing sugars (TRS) content using 1,3-dinitrosalicylic acid (DNS) reagent procedure by Miller ( 1959).The conversion of cellulose and hemicellulose portion of SSB into total reducing sugar were accounted for calculating biomass conversion

percentage, using equation (1) (Choudhary, Umagiliyage, Haddock, 2012).

$$\text{Biomass conversion \%} = \frac{(\text{TRS increase} \times \frac{20}{1000})}{(0.2 \times \frac{C}{100} \times 1.11) + (0.2 \times \frac{H}{100} \times 1.14)} \times 100 \quad \dots (1)$$

Where TRS increase = change in TRS content estimated by the procedure of Miller (1959); C = cellulose content of SSB in %; H = hemicellulose content of SSB in %. The constants 1.11 and 1.14 are reducing sugar conversion factors for cellulose and hemicellulose respectively.

The percentage lignin reduction was calculated according to equation (2).

$$\text{Lignin reduction \%} = \frac{(\text{Lig} - \text{Lpt})}{\text{Lig}} \times 100 \quad \dots (2)$$

Where Lig = lignin content of SSB; Lpt = lignin content of pretreated SSB.

## 2.4. Experimental design and analysis

Response surface methodology (RSM) was used to identify optimal settings of independent variables for each of lime and NaOH pretreatment to maximize biomass conversion and lignin reduction. A three factor central composite design (CCD) for RSM was developed using the automated design of experiments tool, SAS ADX (SAS Institute Inc., Cary, NC).

The independent variables studied were: alkali concentration (% w/v of lime or NaOH), time (h) and SSB loading % (w/v of SSB: alkali solution). The experimental matrix with coded values of independent variables and actual experimental results of dependent variables are included in Table 1. Umagiliyage (2013) contains additional details on the experimental design.

Statistical analysis of experimental data was handled with SAS, version 9.2 (SAS Institute Inc., Cary, NC). The mathematical model for each response was a second order polynomial and the effects of independent variables were described linear, quadratic, and interaction terms. The quality of fit of the model was stated by the coefficient of determination ( $R^2$ ), lack of fit, and its statistical significance. Once a response surface model was fit to the data, contour plots were constructed and canonical or ridge analysis was performed to identify the optimum conditions.

## 2.5. Characterization methods

### 2.5.1. Scanning electron microscopy

Surface morphological characteristics of ground SSB, pretreated SSB and enzymatic hydrolyzed SSB were observed by Scanning Electron Microscopy (SEM) using a Hitachi scanning electron microscope model S-570 (Hitachi Kyowa Engineering Co., Ltd. Minato-ku, Japan).; Prior to the sputter coating with gold palladium, freeze-dried samples were mounted on conductive adhesive tape. The SEM images were obtained at magnifications of 300 – 1500 by utilizing accelerating voltage of 15 to 20 kV.

### 2.5.2. Fourier transform infrared spectroscopy (FTIR)

The Wig-L-Bug ball grinder (PIKE Technologies, Madison, WI) was utilized further size reduction of freeze dried samples (untreated, pretreated and enzymatic hydrolyzed SSB). Then each sample was placed between two KBr discs. Nicolet 6700 FTIR spectrometer (Thermo scientific Inc., Waltham, MA, USA) was used to get FTIR spectra of the samples detained between KBr discs.

## 3. Results and Discussion

### 3.1. Biomass composition

The SSB contained nearly  $36.9 \pm 1.6\%$  cellulose,  $17.8 \pm 0.6\%$  hemicelluloses and  $19.5 \pm 1.1\%$  lignin. The high cellulose and hemicellulose contents of SSB in this study was comparable to other published compositions (Table 2), suggesting that the treated SSB was suitable as lignocellulosic feedstock for biofuel production.

### 3.2. Optimization of lime pretreatment by percentage biomass conversion

The resulting response surface model using the data for effects of lime pretreatment on biomass conversion is shown in equation (3).

$$Y_{11} = 84.56 - 4.84x_1 - 2.99x_2 + 1.72x_3 - 10.9x_1^2 - 4.02x_2^2 - 6.08x_3^2 \quad \dots (3)$$

Where  $Y_{11}$  represent biomass conversion %, as a function of lime concentration ( $x_1$ ), SSB loading ( $x_2$ ) and reaction time ( $x_3$ ).

The overall model ( $H_0: \beta_1 = \beta_2 = \dots \beta_{13} = 0$ ) was highly significant ( $F = 8.7$ ,  $df = 9$ ,  $10$ ,  $P = 0.0011$ ), suggesting that some of the regressors explain significant variation in biomass conversion %. The linear and quadratic term in the model were significant with  $R^2=0.8877$ , indicating the regression model could offer fairly decent predictions. The lack-of-fit was insignificant ( $F = 3.9$ ,  $df = 5$ ,  $10$ ,  $P < 0.08$ ), which suggested that predictions made from a general quadratic model for this experiment can be considered just as accurate as running additional experiments. Parameter optimization, response surface, contour curves and canonical analysis were obtained using this equation. All the independent variables in this model (lime concentration, SSB loading and time) were significant. Canonical analysis suggested that predicted maximum stationary point for biomass conversion was 85.63% at a lime concentration of 1.7%, SSB loading of 6.1 % and time of 2.4 h. The highest significant effect was from lime concentration ( $F=14.77$ ,  $df=4$ ,  $10$ ,  $P=0.0003$ ). SSB loading was the second influential variable and time was the least significant factor influencing TRS yield. Our previous experiments also suggested that longer treatment time did not exert substantial effects on biomass conversion (Liang *et al.*, 2012b; Umagiliyage *et al.*, 2010). Lime pretreatment of sugarcane bagasse also showed simiolar relationship, but the lime loading effect was negative in linear and quadratic terms, indicating it was not necessary to add greater amounts of lime to achieve maximum biomass conversion (Rabelo *et al.*, 2009). SSB loading beyond 6.1 % (w/v) may not increase biomass conversion since available lime might be limited during pretreatment. Another reason could be that compaction of SSB in the reactor reduces accessibility of lime to particle surfaces. This was consistent with previous studies on lime pretreatment of switchgrass (Chang *et al.*, 1997) and sugarcane bagasse (Rabelo *et al.*, 2009).

### 3.3. Optimization of lime pretreatment by lignin reduction

The ANOVA for lignin reduction showed that the overall model was highly significant ( $F = 11.41$ ,  $df = 9$ ,  $10$ ,  $P = 0.0004$ ). In addition, the lack-of-fit was insignificant ( $F = 1.53$ ,  $df = 5$ ,  $10$ ,  $P = 0.325$ ), with R-squared value of 0.9113 supporting fairly good prediction value. ANOVA test on factors showed that only lime concentration ( $F = 16.96$ ,  $df = 4$ ,  $10$ ,  $P = 0.0002$ ) and time ( $F = 8.69$ ,  $df = 4$ ,  $10$ ,  $P = 0.0027$ ) were significant. SSB loading was non-significant ( $F = 1.84$ ,  $df = 4$ ,  $10$ ,  $P = 0.1973$ ) as  $P > 0.05$ , therefore the response surface equation simplified into two factors as in equation (4).

$$Y_{21} = 36.55 + 10.99x_1 + 7.46x_3 - 8.75x_1^2 - 6.64x_3^2 \quad \dots (4)$$

Where  $Y_{21}$  represent lignin reduction % by lime pretreatment as a function of lime concentration ( $x_1$ ), SSB loading ( $x_2$ ).

In the canonical analysis, the model predicted lignin reduction was maximum (44.54 %) when lime concentration = 3.5 % (w/v), SSB loading = 11 % (w/v), and time = 2.4 h. Based on the eigenvalues the most influential variable in the model was lime concentration, followed by time and SSB loading.

Lignin reduction in this study was larger when compared with those for switchgrass. Chang *et al.* (1997) reported best lignin reduction of 29% after 16 h pretreatment under temperature of 100 or 120 °C, lime loading of 0.1 g/g dry biomass, and water loading of 9 ml/g dry biomass. Our results show significant improvements over those of Chang *et al.* (1997) giving higher lignin reduction in shorter time. In a study of poplar wood lime pretreatment (Chang *et al.*, 2001), the removal of lignin was 77.5% (pretreatment conditions: 150°C, 6 h, 0.1 g of  $\text{Ca(OH)}_2$  /g of dry poplar wood, 9 mL of water/g of dry biomass) at raised oxygen pressure that was much higher lignin removal and likely due to the elevated pressure and temperature. So, even though under same chemical pretreatment, the performance of pretreatment for different types of lignocelluloses can differ widely according to composition and severity of pretreatment conditions.

### 3.4. Simultaneous optimization of lime pretreatment by biomass conversion and lignin reduction

The ultimate objective of this study was to determine optimal alkali pretreatment conditions to obtain higher biomass conversion (TRS yield) while achieving higher lignin reduction from SSB. Therefore individual optimization of dependent variables was not adequate. Thus, two quadratic models (equations 3 and 4) obtained by RSM were used for optimization of lime pretreatment using simultaneous maximization of lignin removal as well as biomass conversion.

For simultaneous optimization with the two response surfaces (for  $y_{11}$  and  $y_{21}$ ), optimized area of response variables were visually obtained by overlaying their contour plots (Figure 2). Chang and



Holtzaple (2000) showed “...lime and other alkaline pretreatments increased the accessibility of lignocellulose to enzyme hydrolysis by removing the acetyl and uronic acid substituents that might be present on the hemicellulose fraction of lignocellulose”. By providing higher approachability to the hydrolysable polysaccharides may be due to the lime changing the lignin structure, to facilitate that lignin was solubilized and eroded from SSB, meaning that higher lignin removal may cause higher biomass conversion. This was confirmed by the contour plots in figure 3 showing overlapping of contours of higher lignin reduction with contours of higher biomass conversion value.

The prime objective in any pretreatment process is to release more sugar for microbial fermentation, hence in this study; top five pretreatment conditions were predicted for highest biomass conversion while higher lignin reduction is listed in Table 3. By having several combinations of predicted values for simultaneous optimized responses is important when choosing most convenient and cost-effective condition. Thus cost-effectiveness of pretreatment for industrial conversion of SSB to biofuels would be expected to be optimal at a  $\text{Ca}(\text{OH})_2$  concentration = 1.7 (% w/v), SSB loading = 6.0 (% w/v), and time = 2.4 h

### 3.5. Optimization of lye pretreatment by percentage biomass conversion

The fitted quadratic model for optimization of lye pretreatment is shown in equation (5).

$$Y_{12} = 92.74 - 4.76x_1 - 3.11x_2 + 1.47x_3 - 17.3x_1^2 - 10.53x_2^2 - 11.6x_3^2 \quad (5)$$

Where  $Y_{12}$  represent biomass conversion %, as a function of NaOH concentration ( $x_1$ ), SSB loading ( $x_2$ ) and reaction time ( $x_3$ ).

ANOVA of the regression model for biomass conversion demonstrated that the model (equation 5) was significant ( $F=17.39$ ,  $df=9, 10$ ,  $P < 0.0001$ ). The experimental data fitted the second order polynomial equation well as indicated by high  $R^2$  value of 0.9399. The lack-of-fit was insignificant ( $F = 2.71$ ,  $df = 5, 10$ ,  $P = 0.1491$ ), which suggested that the quadratic response surface model was adequate to make prediction. The linear and quadratic terms in the model were significant, whereas cross product was insignificant. Three independent variables in this model (NaOH concentration, SSB loading and time) were highly significant.

Canonical analysis suggested that predicted maximum was 93.28 % of biomass conversion, when independent variables were NaOH concentration = 1.8 (% w/v), SSB loading = 7.0 (% w/v), and time = 2.1 h. The NaOH concentration was the most responsible factor in lye pretreatment, followed by time and SSB loading respectively.

Goshadrou, Karimi, and Mohammad (2011) was able to get approximately 92% of glucose yield after 72 h of enzymatic hydrolyzed SSB with NaOH pretreatment condition of 12% (w/v) NaOH solution in a 5% (w/v) SSB suspension. Over 90% glucan saccharification yield was reported for SSB pretreated by 0.5 M NaOH with 5% solid to liquid ratio at room temperature for 30 min but when increased chemical concentration nor time neither increased the yield after 1.0M and 60 min whereas Wu et al., (2011) lost large amount of xylan yield while increasing NaOH concentration. Which means after passing over certain level of severity of pretreatment, biomass conversion was diminished, supported our research findings.

### 3.6. Optimization of lye pretreatment by lignin reduction

The fitted quadratic model for optimization of lignin reduction is shown in equation (6).

$$Y_{22} = 49.14 + 11.86x_1 - 1.44x_2 + 8.63x_3 - 20.38x_1^2 - 10.56x_2^2 - 10.63x_3^2 \quad (6)$$

Where  $Y_{22}$ = Lignin reduction %,  $x_1$  = NaOH concentration,  $x_2$  = SSB loading and  $x_3$  = reaction time.

The ANOVA of quadratic regression model for lignin reduction % was highly significant ( $F = 14.74$ ,  $df = 9, 10$ ,  $P = 0.0001$ ). The  $R^2$  value of 0.9299 and non-significant lack-of-fit ( $F = 4.06$ ,  $df = 5, 10$ ,  $P = 0.0751$ ) suggested that the quadratic response surface model was adequate to make prediction. Three independent variables in this model (NaOH concentration, SSB loading and time) were significant. For the lignin reduction also, NaOH concentration had the highest significant effect ( $P < 0.0001$ ) followed by time ( $P=0.0018$ ) and SSB loading ( $P=0.019$ ).

The fitted model predicted lignin reduction was maximum at 53.5% when lye concentration = 2.6 (% w/v), SSB loading = 8.5(% w/v), and time = 2.9 hr. Wu et al. (2011) showed with increasing treatment time (30 to 120min) at low concentration of lye (0.5M), improved the lignin reduction approximately

15% at room temperature in SSB. Alkaline (2% NaOH) and thermal integrated pretreatment (110-230 °C) resulted higher lignin removal and improve accessibility to enzymes (increment of saccharification from 32-90%), however hemicelluloses eroded increasingly (Sun et al., 2015). Even though low temperature (in this study at 100 °C) pretreatment takes more time (with compared to Sun et al 2015) to get higher lignin reduction, it may be more economically feasible and safe to operate.

### **3.7. Simultaneous optimization of lye pretreatment by biomass conversion and lignin reduction**

The ultimate objective of lye pretreatment was to determine optimal pretreatment conditions to obtain higher biomass conversion (TRS yield) while achieving higher lignin reduction from SSB. So, two quadratic models (equations 5 and 6) obtained by RSM were optimized simultaneously. The top five predicted pretreatment conditions for simultaneous maximization of biomass conversion and lignin reduction is listed in Table 4. For lye pretreatment, 2% (w/v) NaOH, 6.8% SSB loading and 2.3 h duration were the optimal levels with predicted biomass conversion and lignin reduction of 92.9% and 50.0%, respectively. The contour plots of simultaneous optimization responses surfaces are shown in Figure 3. The maxima contours of biomass conversion and lignin reduction overlapped indicating successful optimization. This research, lye pretreatment showed higher lignin removal resulting better biomass conversion with compared to lime pretreatment. That finding tallied with previous research on rice straw (Mohamad Remli, Md Shah, Mohamad, & Abd-Aziz, 2014)

### **3.8. Fourier transform infrared spectroscopy (FTIR)**

Figure 4 shows FTIR spectra of untreated, pretreated, and enzymatically catalyzed hydrolysis of SSB in the wavelength region 1900-800  $\text{cm}^{-1}$ . This spectral region was identified as the most prominent spectral absorbance region associated in carbohydrates and lignin ( Corredor, Salazar, Hohn, Bean, Wang, 2009; Pandey, 1999). Due to compositional variations, spectra of samples showed a noticeably different pattern of absorbance. The hemicellulose peaks were clearly identified at 1730-1732  $\text{cm}^{-1}$  in untreated SSB as branched hemicelluloses have carbonyl groups in alkyl esters (Sene, McCann, Wilson, Grinter, 1994). The FTIR spectrum of pretreated samples showed reduced peaks of hemicellulose because it was partially removed by the pretreatment process. After the pretreatment a peak at 1604  $\text{cm}^{-1}$  was clearly appeared and Sene *et al* (1994) reported was due to  $\alpha$ - $\beta$  double bond in lignin. The peak at 1604  $\text{cm}^{-1}$  was lowered in NaOH pretreated SSB after enzymatic hydrolysis, and remained in hydrolyzed lime pretreated SSB. This suggests that both pretreatments were not able to eliminate lignin entirely but higher effectiveness showed with NaOH pretreatment.

IR spectra responsible for lignin (guaiacyl ring) can be seen at 1515 - 1517  $\text{cm}^{-1}$  in untreated SSB, which continued to be present after pretreatment and were even there after hydrolyzed by enzymes. Lignin in SSB composed of syringyl units was obvious by having the peaks around 1315 - 1317  $\text{cm}^{-1}$  as Pandey, (1999) described carbon-oxygen absorption of syringyl ring of lignin produced peaks at 1315  $\text{cm}^{-1}$ . After pretreatment, these peaks became somewhat prominent with removal of soluble and less visible after hydrolysis. This suggests pretreatment was more effective in removing syringyl ring type lignin in sweet sorghum. Compared to bands around 1515 -1517  $\text{cm}^{-1}$ , the bands responsible for syringyl rings (1315 - 1317  $\text{cm}^{-1}$ ) were less visible in sweet sorghum.

FTIR spectral area closer to 1430, 1370-1375, 1159-1162, 1100-1109, 1058-1059 and 897-900  $\text{cm}^{-1}$  were identified as cellulose-related bands (Alriols, Garcia, Llano-ponte, Labidi, 2010; Pandey, 1999). These bands were weak in untreated SSB. After pretreatment, bands appeared well defined in alkali pretreated SSB and 1159-1162, 1058 and 1034-1035  $\text{cm}^{-1}$  bands appeared stronger in lye pretreated SSB compared to lime pretreated samples. This suggests that lye pretreatment enriched the SSB with cellulose. Cellulose was uncovered as a result of the pretreatment could be evident by the prominence appearing of cellulose related peaks in pretreated SSB. Crystalline-celluloses showed a diluted peak (1098-1109  $\text{cm}^{-1}$ ) even after hydrolyzed with enzymes in both lime treated and lye treated samples. Furthermore, an amorphous-celluloses bands (897-900  $\text{cm}^{-1}$ ) appeared more declining after enzyme hydrolysis in both the pretreatments, denoting that cellulose of amorphous type was more intensely hydrolyzed with enzymes but crystalline cellulose was not hydrolyzed completely.

An asymmetric carbon-oxygen-carbon vibration could be clearly identified in pretreated SSB (1159-1162  $\text{cm}^{-1}$ ). Since cellulases break down  $\beta$  1-4 glycoside bonds in cellulose, those peaks strength



was declined in hydrolyzed SSB. The peak appearing about 1370-1375  $\text{cm}^{-1}$  in untreated samples was as a result of carbon and hydrogen bond distortion (symmetrically) of celluloses (Alriols *et al.*, 2010; Corredor *et al.*, 2009; Pandey, 1999). Those prominent bands almost disappeared after hydrolyzed with enzymes. Pandey, (1999) and Corredor *et al.*, (2009), were assigned the spectral bands 1058  $\text{cm}^{-1}$  and 1035  $\text{cm}^{-1}$  to carbon-oxygen stretching of cellulose and seem to be well defined after pretreatments in this research, but completely disappeared after enzymatic hydrolysis. This confirmed that alkali pretreatment was efficient in exposing cellulose to enzymatic hydrolysis and breaking cellulose to its monomeric sugars for fermentation.

### 3.9. Morphological changes

Morphological features of untreated SSB and alkali pretreated SSB are shown in Figure 5. Untreated SSB exhibited rigid and ordered fibrils with some deposits on the exterior layer (Figure 5a). This outer most layer was observed with different kind of polysaccharides, waxes, lignin, and different binding materials, which tallied with previously reported in corn stover (Kim, Lee, Sunwoo, Kim, 2006), and sorghum leaves and stems (Corredor *et al.*, 2009). In the pretreated SSB, the surface layer was removed and the cell wall was distorted, resulting in exposure of internal structures (Figure 5b, and 5c) which was consistent with observations of SSB structure by Goshadrou, et al., (2011). Even untreated SSB showed some fractures in cell walls because of the squeezing of sweet sorghum stem for expressing juice with roller mills (Figure 12a). Because the cell walls of SSB are already broken to some extent, intensity of pretreatment could potentially be lowered. Differences between lime and lye pretreatment could not be clearly identified in SEM image. Removal of surface wax and other depositions after pretreatment was in agreement with weaker FTIR bands at 1242-1247  $\text{cm}^{-1}$  and 1732  $\text{cm}^{-1}$  after pretreatment. The disrupted SSB structure created by both the pretreatments caused total exposure of internal structure and fibrils to enzymes for efficient saccharification (Figures 5b and c, 6a).

Figure 6 shows SEM images of pretreated SSB before and after enzymatic hydrolysis. The pretreatment disrupted cell walls of SSB and increased the surface area for more accessibility to enzymes (Figure 6b), which tallied with Ma et al (2014). It was observed that some amount of cell wall material (cellulose and hemicellulose) was dissolved and swelled after enzymatic hydrolysis (Figure 6b). The internal fiber structure can be identified with one cell top of another cell to make fibers and then tightly organized fiber cluster, and the remaining structures could be crystalline forms of cellulose, lignin and minerals. Previous research showed cellulose remained as crystallized aggregates after pretreatment (Corredor *et al.*, 2009), suggesting enzymatic hydrolysis had caused degradation of most of the cellulose, leaving solid residues that might be used for other industrial purposes.

### 4. Conclusions

The primary goal of this study was to determine optimal alkali pretreatment conditions to obtain high biomass conversion (TRS yield) while achieving high lignin reduction for biofuel production from SSB. The optimal conditions for lime pretreatment were 1.7 % (w/v) lime concentration, 6.0 % (w/v) SSB loading and 2.4 h time with predicted yields of 85.6% total biomass conversions and 35.5% lignin reduction. For NaOH pretreatment, 2% (w/v) alkali, 6.8% SSB loading and 2.3 h duration was the optimal level with predicted biomass conversion and lignin reduction of 92.9% and 50.0%, respectively. Alkali pretreatment was effective in removing lignin from SSB. Some hemicellulose and a small amount of cellulose were also removed which was consistent with previous studies. More intensive pretreatment conditions such as higher alkali loading and longer time removed higher amounts of hemicelluloses and cellulose.

Supplementary enhancement of this process could emphasis on diminishing sugar degradation and make best use of higher sugar yield to increase efficiency of microbial fermentation. Moreover, integrated financial feasibility of the entire biofuel production process is needed to evaluate the economic profitability of any pretreatment process.

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**Figure Caption:**

Figure 1. Pretreatment incubation setup showing reactor tubes mounted on the rotating drum inside oven.

Figure 2. Overlaid line contours of predicted responses for lime pretreatment. X1= Alkali concentration % w/v; X2= SSB loading % w/v; X3= Reaction time (h)

Figure 3. Overlaid line contours of predicted responses of biomass conversion and lignin reduction in NaOH pretreatment. X1= Alkali concentration % w/v; X2= SSB loading % w/v; X3= Reaction time (h)

Figure 4. FTIR spectra of different SSB in the region of 800-1900  $\text{cm}^{-1}$ .

Figure 5. SEM images of SSB (a- untreated SSB 500x, b- lime pretreated 1h 500x, NaOH pretreated 1h)

Figure 6. SEM images of NaOH pretreated SSB (2% NaOH, 7.5%SSB, 2h) (a- pretreated only, b- pretreated and enzymatic hydrolyzed)



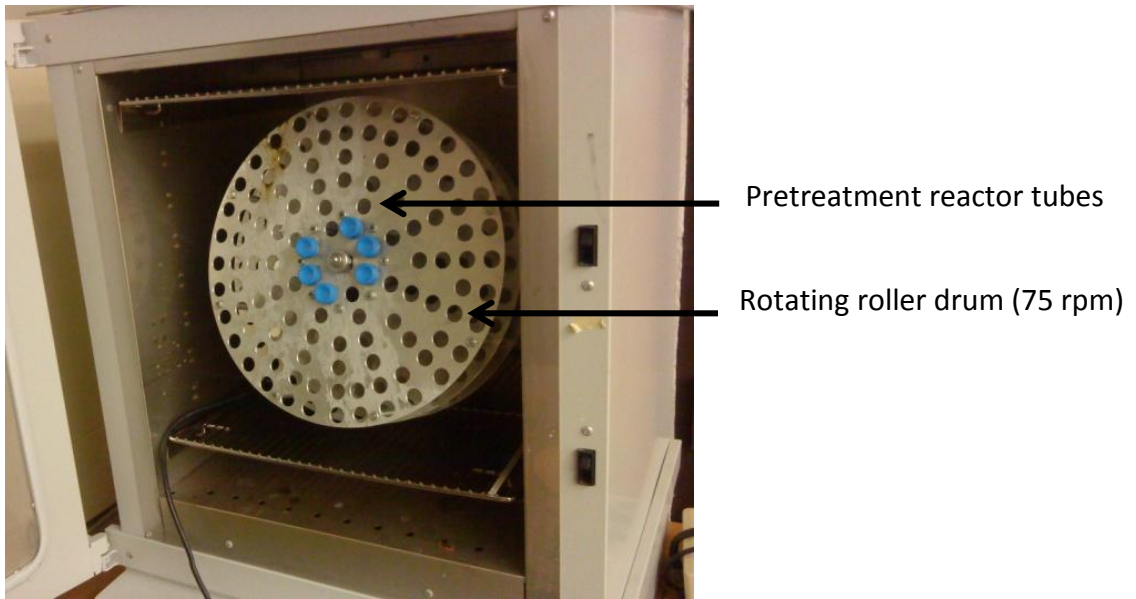


Figure 1. Pretreatment incubation setup showing reactor tubes mounted on the rotating drum inside oven

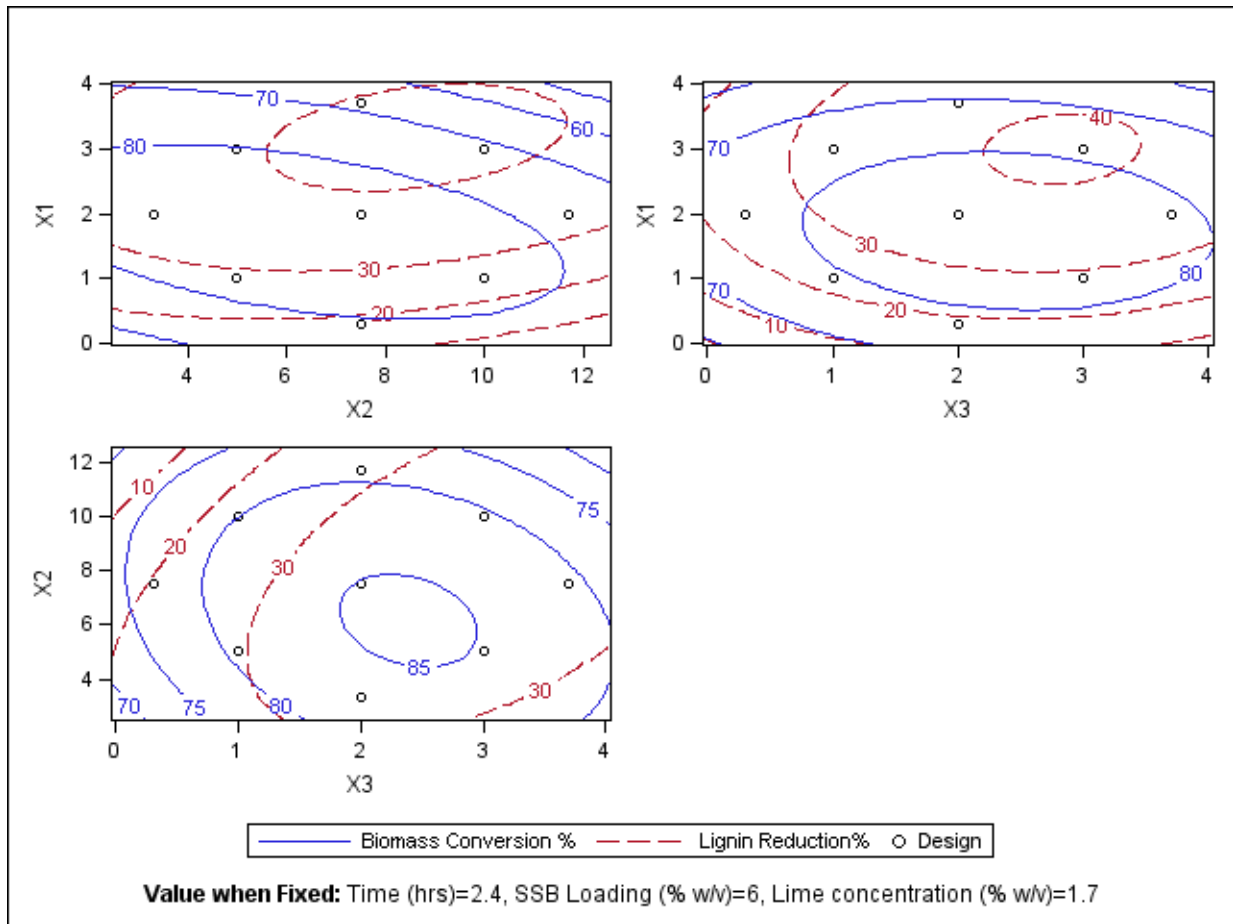


Figure 2. Overlaid line contours of predicted responses for lime pretreatment. X1= Alkali concentration % w/v; X2= SSB loading % w/v; X3= Reaction time (h)

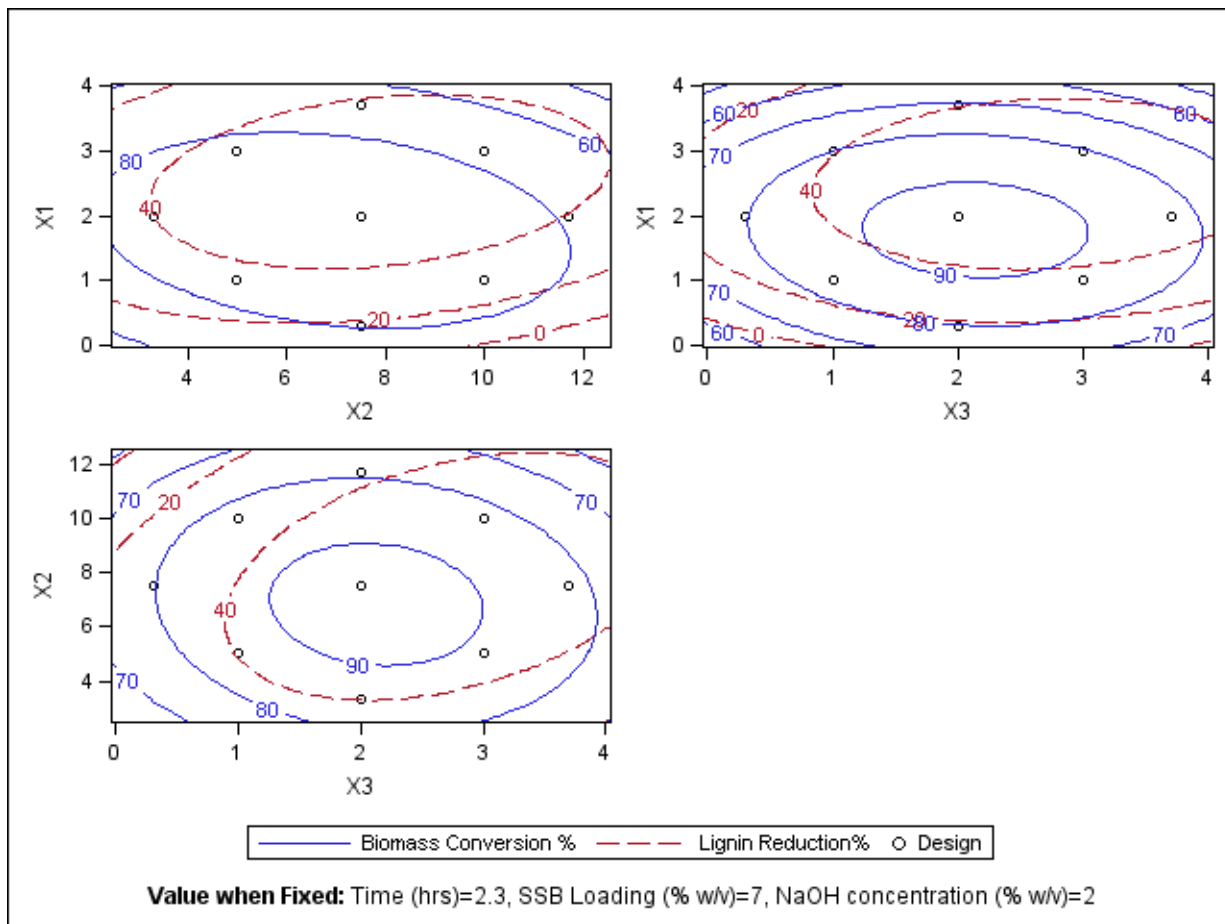


Figure 3. Overlaid line contours of predicted responses of biomass conversion and lignin reduction in NaOH pretreatment. X1= Alkali concentration % w/v; X2= SSB loading % w/v; X3= Reaction time (h)

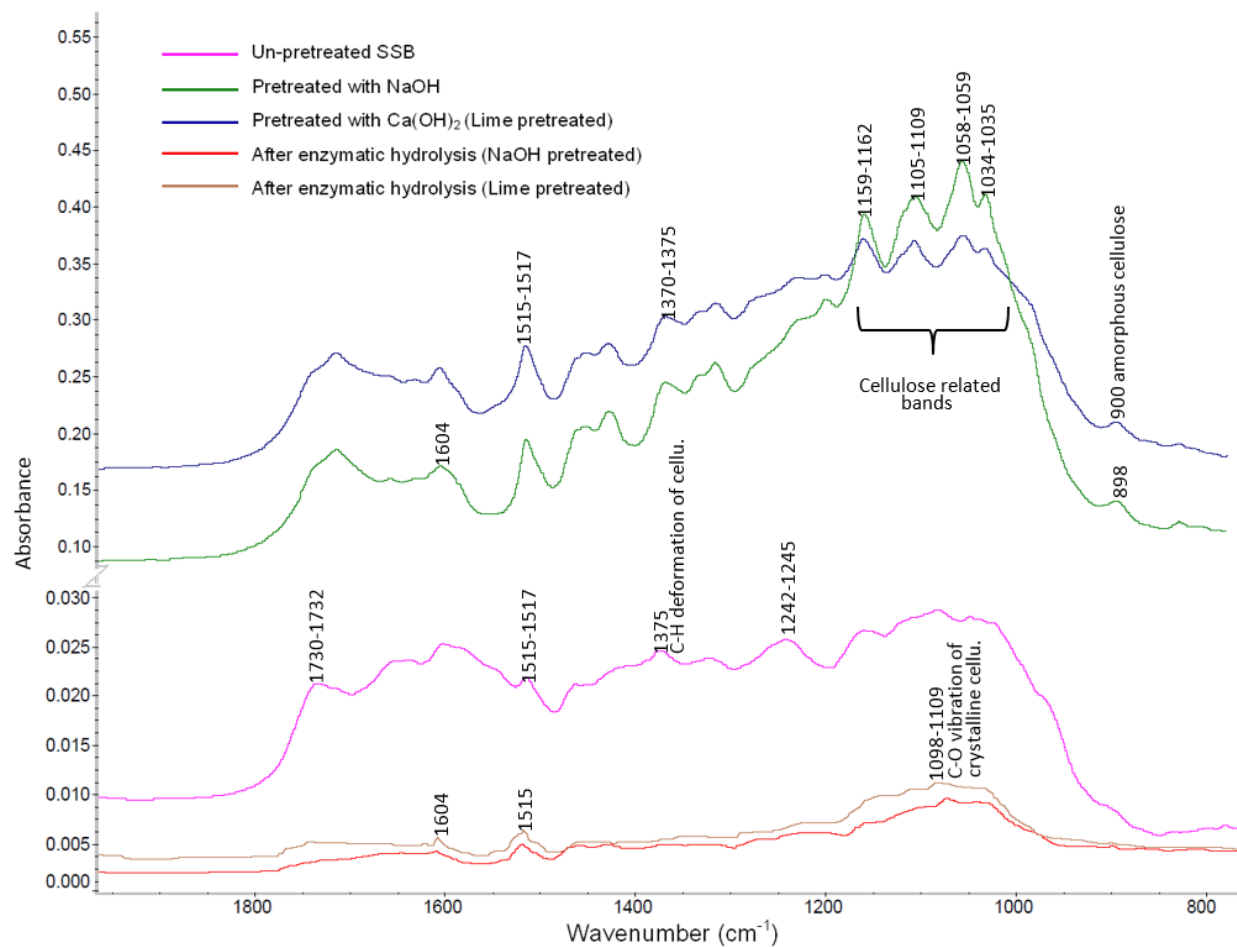


Figure 4. FTIR spectra of of SSB, pre-treated and enzymatically hydrolyzed samples in the region of 800-1900 cm<sup>-1</sup>.

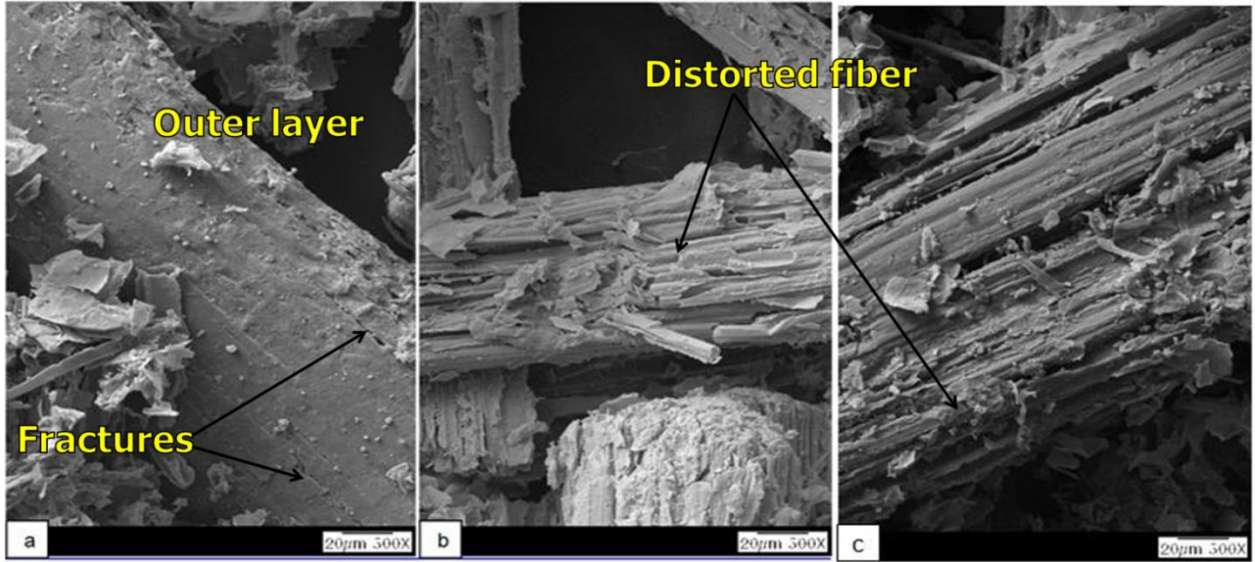


Figure 5. SEM images of SSB (a- untreated SSB 500x, b- lime pretreated 1h 500x, NaOH pretreated 1h)

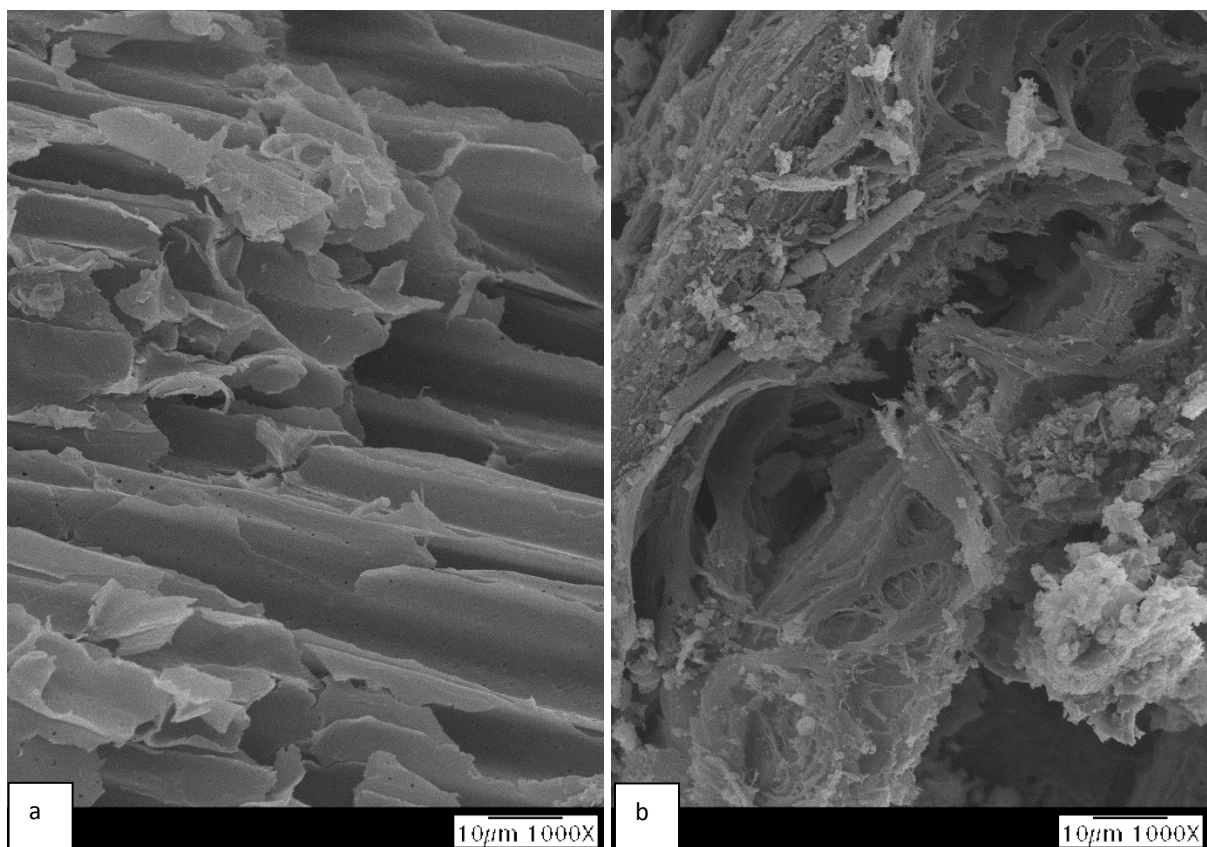


Figure 6. SEM images of NaOH pretreated SSB (2% NaOH, 7.5%SSB, 2h). a- pretreated only, b- pretreated and enzymatic hydrolyzed.

Table 1. Oven alkali pretreatment experimental matrix and results

No.	Coded independent variables			Actual experimental values			Lime pretreatment results		NaOH pretreatment results	
	$x_1$	$x_2$	$x_3$	Alkali conc. % (w/v)	SSB loading % (w/v)	Time (h)	Biomass conversion % ( $Y_{11}$ )	Lignin reduction % ( $Y_{21}$ )	Biomass conversion % ( $Y_{12}$ )	Lignin reduction % ( $Y_{22}$ )



1	-1	-1	-1	1.0	5.0	1.0	77.38	22.62	79.12	29.23
2	-1	-1	1	1.0	5.0	3.0	79.10	25.54	80.93	32.59
3	-1	1	-1	1.0	10.0	1.0	78.42	15.15	80.18	15.99
4	-1	1	1	1.0	10.0	3.0	83.18	24.11	84.87	30.18
5	1	-1	-1	3.0	5.0	1.0	73.82	32.27	75.69	33.88
6	1	-1	1	3.0	5.0	3.0	79.38	36.28	80.87	41.05
7	1	1	-1	3.0	10.0	1.0	73.22	28.69	74.33	30.02
8	1	1	1	3.0	10.0	3.0	68.90	41.05	70.55	48.04
9	-1.68	0	0	0.3	7.5	2.0	79.34	18.77	80.78	16.56
10	1.68	0	0	3.7	7.5	2.0	69.62	41.23	72.02	46.63
11	0	-1.68	0	2.0	3.3	2.0	86.84	36.28	88.73	41.23
12	0	1.68	0	2.0	11.7	2.0	75.92	33.34	77.64	41.73
13	0	0	-1.68	2.0	7.5	0.3	77.46	22.62	80.92	33.34
14	0	0	1.68	2.0	7.5	3.7	81.14	41.59	83.27	49.34
15	0	0	0	2.0	7.5	2.0	84.28	39.87	94.82	53.22
16	0	0	0	2.0	7.5	2.0	82.79	37.20	90.72	50.59
17	0	0	0	2.0	7.5	2.0	83.71	32.68	93.14	46.59
18	0	0	0	2.0	7.5	2.0	86.48	33.34	93.98	48.26
19	0	0	0	2.0	7.5	2.0	86.45	36.68	90.08	48.59
20	0	0	0	2.0	7.5	2.0	83.44	38.92	93.44	46.82

Table 2. Composition of sorghum biomass from various sources

<b>Biomass</b>	<b>Cellulose %</b>	<b>Hemicellulose %</b>	<b>Lignin %</b>	<b>Reference</b>
Forage sorghum(MSU)	35.6 ± 0.8	18.4 ± 0.5	18.2 ± 0.2	Li <i>et al.</i> , 2009
SSB at Homer, LA	44.5	30.5	22	Salvi <i>et al.</i> , 2010
SSB obtained in Hungary	36.3	27.7	18.6	Sipos <i>et al.</i> , 2009
SSB (MSU)	38.3 ± 0.3	18.2 ± 0.1	19.7 ± 1.1	Li <i>et al.</i> , 2009
SSB (SIU) <sup>1</sup>	36.9 ± 1.6	17.8 ± 0.6	19.7 ± 1.1	This study

<sup>1</sup>NREL procedure,

Table 3. Top five predictions for simultaneous optimization of lime pretreatment

Obs.	<b>Lime conc.</b> % (w/v) (x <sub>1</sub> )	SSB Loading % (w/v) (x <sub>2</sub> )	Time (h) (x <sub>3</sub> )	Biomass conversion % (Y <sub>11</sub> )	Lignin reduction% (Y <sub>21</sub> )
1	1.7	6.2	2.4	85.63	35.58
2	1.7	6.1	2.4	85.63	35.54
3	1.7	6.3	2.4	85.62	35.61
4	1.7	6.0	2.4	85.62	35.50
5	1.8	6.0	2.4	85.62	36.24

Table 4. Top five predictions for simultaneous optimization of lye treatment

Obs.	NaOH conc. (% w/v) ( $x_1$ )	SSB Loading (% w/v) ( $x_2$ )	Time (h) ( $x_3$ )	Biomass conversion % ( $Y_{12}$ )	Lignin reduction% ( $Y_{22}$ )
1	2	6.8	2.3	92.9277	50.0485
2	2	6.9	2.3	92.9220	50.1246
3	2	7.0	2.3	92.9044	50.1887
4	2	7.3	2.2	92.8881	50.0073
5	2	7.1	2.3	92.8748	50.2408