Optimization of Dilute NaOH Pretreatment at Mild Temperatures for Monomeric Sugar Release from Sorghum Pith Using Response Surface Methodology

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Response surface methodology (RSM) was used to optimize the alkali pretreatment conditions for maximum fermentable sugar yield from sorghum pith with respect to NaOH concentration (0.5% to 2%), reaction temperature (20 °C to 40 °C), and pretreatment time (2 h to 20 h). The pretreatment caused a slight loss of glucan, but a significant removal of lignin and xylan from the pith, particularly lignin. The optimized results showed that the pretreatment conditions for the maximum predicted enzymatic glucose yield (90.5%) were 19.5 h, 2% NaOH, and 40 °C, while that for the maximum predicted enzymatic xylose yield (57.7%) were 9.9 h, 1.4% NaOH, and 37.5 °C. The optimized pretreatment conditions for the maximum total sugars yield were 16.2 h, 2% NaOH, and 37 °C, under which 72.4% of the glucan and xylan present in the raw material were experimentally hydrolyzed to release monomeric sugars. Additionally, with the optimized combination of 15 FPU cellulase and 7.5 CBU β-glucosidase per gram of pretreated material, the saccharification efficiency approached 90% glucan and 74% xylan present in the pretreated material (obtained at 37 °C for 16 h with 2% NaOH). This study may provide useful information for the development of novel alternative feedstocks for cellulosic ethanol production.

Keywords: Sorghum pith; NaOH pretreatment; Enzymatic hydrolysis; RSM

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INTRODUCTION

Lignocellulosic biomass, which is comprised of cellulose, hemicelluloses, and lignin, is a promising material for bioethanol production. The hemicellulose-lignin complex and crystalline structure of cellulose make it recalcitrant to enzymatic attack; therefore, lignocellulosic biomass has poor yields of fermentable sugars (Wu *et al*. 2011; Grimaldi *et al*. 2015; Huang *et al*. 2015; Ma *et al*. 2015). To make lignocellulose more susceptible to subsequent enzymatic hydrolysis, an efficient pretreatment process is required. A range of pretreatment methods have been extensively examined to process various biomass feedstocks to release sugars, including acid and alkali, ammonia fiber explosion, liquid hot water, organosolv, steam explosion, microwaves, and ionic liquid-based pretreatments (Alizadeh *et al*. 2005; Li *et al*. 2010; Park *et al*. 2010; Horn *et al*. 2011; Choudhary *et al*. 2012; García *et al*. 2014; Wilkinson *et al*. 2014; Xiao *et al*. 2014). However, these alternative methods are not satisfactory because of their intrinsic drawbacks, such as a high energy usage and cost, low conversion efficiency, environmental pollution, and technological impasses. Therefore, an appropriate pretreatment process applied to a specific lignocellulosic material is of vital importance to make bioconversion processes more economical and efficient.

Sorghum bicolor is an energy crop with a high photosynthetic efficiency and a short growth cycle of 3 months to 4 months (Cao *et al*. 2012; Wang *et al*. 2013). It can be planted under various climate conditions (Wang *et al*. 2013). Because of its high yields of fermentable sugars and biomass, *S. bicolor* is regarded as an herbaceous energy crop that is attractive and promising for fuel ethanol production (Li *et al*. 2014). The sorghum stem internodes consist of rind and pith that differ in their structure and composition. The pith is dominated by wide parenchyma cells, whereas the rind fraction consists of vascular bundles containing thick-walled fibers (Hatfield *et al*. 1999). This suggests that the pith fraction is much less recalcitrant than the rind fraction.

Compared with other pretreatment methods, alkali pretreatment has the advantages of simple devices, convenient operation, and low formation of fermentation inhibitors (Umagiliyage *et al*. 2015; Liu *et al*. 2016). The major effect of alkali pretreatment is the removal of a large portion of lignin and acetyl groups, and various uronic acid substitutions on the hemicellulose, which increases the accessible surface area for increasing the biomass reactivity and results in an enhancement of the enzymatic hydrolysis efficiency (Cabrera *et al*. 2015; Gabhane *et al*. 2015). Sodium hydroxide (NaOH) is one of the most effective alkaline reagents for the pretreatment of various herbaceous biomasses, such as switchgrass (Gupta and Lee 2010; Xu and Cheng 2011), corncob (Baadhe *et al*. 2014), corn stover (Cui *et al*. 2012; Lai *et al.* 2017), and rice straw (Ibrahim *et al*. 2011; Kim and Han 2012). The optimum pretreatment conditions vary depending on the materials used. Pretreatment of sorghum stem using NaOH has been tentatively developed (McIntosh and Vancov 2010; Wu *et al*. 2011; Nikzad *et al*. 2014). However, either high reaction temperatures or NaOH loadings have been utilized; although, the required pretreatment time was relatively short in these studies.

This study focused on assessing the pretreatment ability of sorghum pith with dilute NaOH at low temperatures and optimizing the conditions for achieving a maximum enzymatic hydrolysis yield of monomeric sugars (glucose and xylose) using response surface methodology (RSM). Furthermore, the optimum enzyme dosage was identified to promote production. In comparison with previous work, this study provided a reference for the current development of the production of fermentable sugars from lignocelluloses under mild pretreatment condition.

EXPERIMENTAL

Materials

Sorghum stem (Lunuo No. 08) was gathered from Zhuji, Zhejiang, China. After separation from the outer rind layer by hand peeling using sharp knives, the pith sample was beaten into a powder with a pulverizer (Taikete Technology Co., Ltd, Tianjin, China), which was followed by repeated washings with water. Subsequently, the washed material was put into a drying oven at 50 °C, dried to a constant weight, and then stored at room temperature in air-tight zip lock bags for future use.

Commercial β-glucosidase from *Aspergillus niger* (Novozym188) and a *Trichoderma reesei* cellulase preparation (C2730) were purchased from Sigma-Aldrich (St

Louis, USA). The β-glucosidase, xylanase, and filter paper activities of the cellulase preparation (C2730) were 22.2 U/g, 239 U/g, and 117 FPU/g, respectively. The β-glucosidase preparation (Novozym188) had very little xylanase or filter paper activity and had a β-glucosidase activity of 269 U/g.

NaOH Pretreatment and Enzymatic Hydrolysis

A dilute NaOH aqueous solution (0.5% to 2%, w/v) was used to treat 1 g of material with a solid to liquid ratio of 1:20 (w/v). The pretreatment was conducted in triplicate at 20 °C to 40 °C in a static water bath and the residence times were 2 h to 20 h. The pretreated solid was separated from the alkali liquor by vacuum filtration. The solid fraction was repeatedly washed with water until the pH was neutral, and then it was freeze-dried for the subsequent compositional analysis and enzymatic hydrolysis experiments.

Enzymatic hydrolysis was performed in 50-mL conical flasks. Cellulase (15 FPU/g of glucan) and *β*-glucosidase (30 CBU/g of glucan) were added to the substrate. Phosphate buffer (pH = 4.8) was used to get a total working volume of 20 mL. Sodium azide (10 mM) was supplemented to prevent microbes from growing in the hydrolysate. The enzymatic hydrolysis was conducted in an incubator at 50 °C and shaken at 150 rpm for 72 h. Samples of the hydrolysates were taken during the course of testing and clarified by centrifuging them at 10000 rpm for 5 min. High-performance liquid chromatography (HPLC) was used to analyze the glucose and xylose in the supernatants with the method mentioned below.

The lignin, glucan, and xylan recoveries, the lignin removal, and the enzymatic yields of the glucose, xylose, and total sugars were calculated with Eqs. 1 to 7,

$$\text{Lignin recovery (\%)} = \frac{\text{Amount of lignin in the pretreated solid (g)}}{\text{Amount of lignin in the raw material (g)}} \times 100\% \tag{1}$$

$$\text{Glucan recovery (\%)} = \frac{\text{Amount of glucan in the pretreated solid (g)}}{\text{Amount of glucan in the raw material (g)}} \times 100\% \tag{2}$$

$$\text{Xylan recovery (\%)} = \frac{\text{Amount of xylan in the pretreated solid (g)}}{\text{Amount of xylan in the raw material (g)}} \times 100\% \tag{3}$$

$$\text{Lignin removal (\%)} = \frac{\text{Amount of lignin in the raw material (g)} - \text{Amount of lignin in the pretreated solid (g)}}{\text{Amount of lignin in the raw material (g)}} \times 100\% \tag{4}$$

$$\text{Glucose yield (\%)} = \frac{0.9 \times A_G\ \text{(g)}}{\text{Amount of glucan in the raw material (g)}} \times 100\% \tag{5}$$

$$\text{Xylose yield (\%)} = \frac{0.88 \times A_X\ \text{(g)}}{\text{Amount of xylan in the raw material (g)}} \times 100\% \tag{6}$$

$$\text{Total sugar yield (\%)} = \frac{0.9 \times A_G\ \text{(g)} + 0.88 \times A_X\ \text{(g)}}{\text{Total amount of glucan and xylan in the raw material (g)}} \times 100\% \tag{7}$$

where A_G is the amount (%) of the released glucose after enzymatic hydrolysis, and A_X is the amount (%) of released xylose after enzymatic hydrolysis. The conversion factors for the dehydration or polymerization to glucan and xylan were 162/180 (0.9) for glucose and

132/150 (0.88) for xylose.

As for the effect of the enzyme loading on enzymatic hydrolysis of the pretreated sorghum pith (obtained at 37 °C for 16 h with 2% NaOH), pretreated samples (1 g dry weight) underwent enzymatic hydrolysis in 50-mL conical flasks containing 50 mM phosphate buffer (pH = 4.8) at 5% (w/v) solid loading. Different loadings of cellulase and *β*-glucosidase were used for the enzymatic hydrolysis. The flasks were incubated at 50 °C and shaken at 150 rpm for 72 h.

Analytical Methods

Chemical compositional analysis of the raw and pretreated materials

National Renewable Energy Laboratory methods (Sluiter *et al*. 2008) were used to determine the chemical composition of the raw and pretreated sorghum pith. First, a 0.3-g sample was soaked in 3 mL of 72% (w/w) H_2SO_4 at 30 °C for 60 min, which was followed by the addition of distilled water to dilute the H_2SO_4 concentration to 4% (w/w). Then, the mixture was autoclaved at 121 °C for 60 min. The amounts of glucose, xylose, arabinose, and galactose in the acid hydrolysate were determined with HPLC (Agilent 1100, Palo Alto, USA). The HPLC system was equipped with a refractive index detector and Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm, Hercules, USA). The column temperature was 55 °C. The flow rate of the mobile phase (5 mM H_2SO_4) was 0.6 mL/min. The acid-soluble lignin content was detected using ultraviolet visible absorbance at 280 nm, and the acid-insoluble lignin content was determined gravimetrically. The ash content was determined after incineration of an aliquot of the material at 550 °C.

Scanning electron microscopy

Micrographs were taken using field emission scanning electron microscopy (SEM) (FEI, Quanta 200, Hillsboro, OR, USA). Before imaging, the samples were coated with a thick layer of gold to make the samples conductive, which avoided excessive buildup of charge on the samples. The accelerating voltage was 15 kV, and the energy resolution was 130 eV.

X-ray powder diffraction analysis

An X-ray diffraction (XRD) diffractometer (Rigaku, Ultima IV, Japan) was employed to record the X-ray diffractograms from the diffraction angles (2θ) of 5° to 50° at a scanning speed of 5°/min. The following equation (Segal *et al*. 1959) was used to determine the crystallinity index (*CrI*),

$$CrI\ (\%) = (I_{002} - I_{18^\circ}) \times 100 / I_{002} \tag{8}$$

where I_{002} is the intensity of the diffraction peak at 22° and I_{18° is the intensity attributed to the amorphous region at a 2θ of 18°.

Experimental Design

A Box-Behnken design containing three independent variables was employed to optimize the NaOH pretreatment conditions for sorghum pith, which included the alkali loading (%, w/v), time (h), and temperature (°C). Levels of independent variables were selected on the basis of the preliminary studies to ensure an appropriate range of responses (Table 1). The enzymatic hydrolysis yields of glucose, xylose, and total sugars, and the lignin removal rate were chosen to analyze the responses. Arabinose was not considered in the mathematical model because it was not detected in the enzymatic hydrolysate. All of the 17 experimental runs (including five replicates at central points) were designed with Design-Expert 8.0.6 (Stat Ease, Inc., Minneapolis, USA). Each point was done in triplicate, and the average of the data was reported. The estimation of the effect that the conjunct independent variables had on each response was determined by regression analysis. Coefficients of determination (R^2) were calculated by the quadratic polynomial model. Additionally, an analysis of variance (ANOVA) was employed to statistically analyze the models. The models were evaluated by comparing the R^2 and adjusted R^2. The statistical significance was determined with F- and p-values. Response surfaces were created to illustrate the effects of the independent variables on the responses according to the fitted quadratic polynomial model.

Table 1. Independent Variable Ranges in the Experimental Design

Coded Level of the Factors	Temperature (°C)	NaOH Loading (%, w/v)	Time (h)
Low Level (-1)	20	0.5	2
Central Level (0)	30	1	11
High Level (+1)	40	2	20

RESULTS AND DISCUSSION

Composition of the Raw Material

The chemical composition of the sorghum pith determined in this study is shown in Table 2. The main component in the raw biomass was glucan, which was followed by xylan and lignin. The glucan and xylan together accounted for 61% of the sorghum pith, which attested to the richness of polysaccharides in this biomass. Other minor hemicellulosic sugar polymers were present, namely arabinan and galactan, but they were scarce. Billa *et al*. (1997) reported that sweet sorghum pith contains approximately 59.2% glucan and 15.1% xylan. The differences between these contents reported in this study and the literature may have been because of the cultivar type, climate, location of cultivation, and other factors (McIntosh and Vancov 2010).

Table 2. Major Components of the Sorghum Pith

Composition	Percentage (%)*
Glucan	39.29 ± 0.08
Xylan	21.67 ± 0.24
Arabinan	4.60 ± 0.12
Galactan	0.92 ± 0.01
Acid insoluble lignin	20.34 ± 0.31
Acid soluble lignin	0.50 ± 0.01
Ash	3.83 ± 0.38
Other	8.85
***Calculated on a dry-weight basis**	

Effect of the Alkali Pretreatment on the Glucan, Xylan, and Lignin Recoveries

Figure 1 displays the glucan, xylan, and lignin recoveries with respect to the initial contents in the sorghum pith after pretreatment with different NaOH concentrations (0.5% to 2%) at 20 °C to 40 °C for 2 h to 20 h. The pretreatment led to a slight glucan loss, and the glucan recoveries of most of the runs were more than 90%. Similar observations were reported by Wu *et al.* (2011), who studied the pretreatment of sweet sorghum bagasse with NaOH and where approximately 95% of the glucan present in the raw material was recovered after pretreatment. In contrast, the pretreatment process had an obvious effect on the degradation and removal of xylan, and a rapid increase in the removal rate was observed with an increasing pretreatment severity, especially for the alkali loading. For instance, pretreatment for 11 h at 40 °C with 0.5% NaOH resulted in an approximately 18% loss of xylan in the pith, whereas the loss rate sharply increased to 52% when the pith was treated with 2% NaOH at the same temperature and reaction time. The lignin was generally effectively solubilized and removed at the NaOH loadings of 1.25% and 2%, and most of the lignin recoveries were below 50%. In contrast, relatively high recoveries of lignin (more than 50%) were found when the alkali loading was 0.5%.

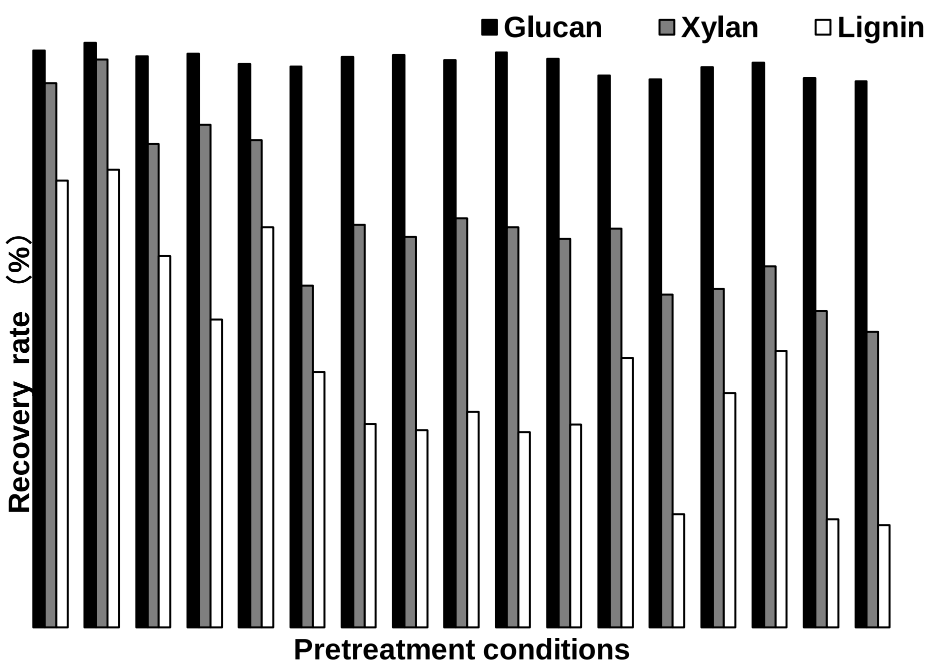

Fig. 1. Recovery of glucan, xylan, and lignin from the sorghum pith with different pretreatment conditions

Model Fitting

The enzymatic hydrolysis yields of the glucose, xylose and total sugars, and the lignin removal for different independent variables are listed in Table 3. Each response is the average of three replicates. The glucose, xylose and total sugar yields, and the lignin removal ranged from 29.1% to 83.1%, 23.8% to 56.7%, 27.8% to 70.6%, and 25.1% to 83.2%, respectively. For a control sample, some pith was soaked in distilled water at 40 °C for 20 h. The enzymatic glucose and xylose yield of the water-treated pith were only 8.0% and 10.0%, respectively.

Table 3. Box-Behnken Experimental Data and Corresponding Glucose, Xylose, and Total Sugars Yields, and Lignin Removal

Run	Experimental Factor			Lignin Removal (%)	Glucose Yield (%)	Xylose Yield (%)	Total Sugar Yield (%)
	X_1	X_2	X_3				
1	11	1.25	30	69.15	66.76	55.46	62.1
2	11	1.25	30	65.74	64.38	56.73	62.83
3	11	0.5	40	49.62	37.99	42.47	39.61
4	20	1.25	20	58.19	52.11	45.93	49.88
5	20	2	30	82.34	82.62	44.94	70.57
6	11	1.25	30	67.99	67.17	54.38	61.27
7	11	1.25	30	67.92	63.81	53.98	63.86
8	2	1.25	40	55.91	53.51	53.69	53.58
9	20	1.25	40	81.49	72.52	50.95	64.74
10	11	2	20	61.66	62.99	44.98	56.5
11	20	0.5	30	39.26	34.17	37.4	35.34
12	2	1.25	20	34.57	34.72	34.86	34.77
13	2	0.5	30	25.13	30.07	23.81	27.81
14	11	0.5	20	26.97	29.12	25.51	27.82
15	11	1.25	30	66.72	65.85	54.13	62.1
16	2	2	30	54.78	58.29	48.61	54.8
17	11	2	40	83.25	83.12	48.42	69.97

X_1 – Time (h); X_2 – Alkali loading (%); X_3 – Temperature (°C)

The lignin removal and glucose, xylose, and total sugars yields were successfully modeled using linear regression, based on the pretreatment conditions. The ANOVA of the models for the glucose, xylose, and total sugars yields, and the lignin removal are summarized in Table 4. All of the models were significant at a p less than 0.0001, which indicated that the models were significant with high confidence levels. None of the lack-of-fit values were significant, which implied the established models were adequate for prediction. Although some model terms were insignificant ($p > 0.05$), these factors were not excluded to support the hierarchy of the models. The R^2 was 0.9969 for the lignin removal, 0.9938 for the glucose yield, 0.9919 for the xylose yield, and 0.9955 for the total sugars yield. The adjusted R^2 was 0.9929 for the lignin removal, 0.9859 for the glucose yield, 0.9815 for the xylose yield, and 0.9896 for the total sugars yield. The predicted R^2 was 0.9692 for the lignin removal, 0.9268 for the glucose yield, 0.9181 for the xylose yield, and 0.9439 for the total sugars yield. The high values of both R^2 values and adjusted R^2 values indicated a good fit of the models to experimental data (Mohammadi *et al*. 2014). The predicted R^2 values were all in reasonable agreement with their adjusted R^2 values, which also desirably confirm the significance of these models (Cheng *et al*. 2011). The following equations are the final empirical quadratic models that took the actual factors into consideration:

$$\textit{Lignin removal}\ (\%) = -62.104 + 2.509X_1 + 61.967X_2 + 2.585X_3 + 0.497X_1X_2 + 0.005X_1X_3 - 0.035X_2X_3 - 0.092X_1^2 - 17.148X_2^2 - 0.025X_3^2 \quad (9)$$

$$\textit{Glucose}\ (\%) = -53.579 + 1.784X_1 + 38.035X_2 + 3.442X_3 + 0.749X_1X_2 + 4.5 \times 10^{-3}X_1X_3 + 0.375X_2X_3 - 0.089X_1^2 - 12.637X_2^2 - 0.052X_3^2 \quad (10)$$

$$Xylose\ (\%) = -84.542 + 3.593X_1 + 79.642X_2 + 3.615X_3 - 0.639X_1X_2 - 0.038X_1X_3 - 0.451X_2X_3 - 0.063X_1^2 - 19.785X_2^2 - 0.035X_3^2 \quad (11)$$

$$Total\ sugar\ (\%) = -70.515 + 2.406X_1 + 54.216X_2 + 3.891X_3 + 0.305X_1X_2 - 0.011X_1X_3 + 0.056X_2X_3 - 0.080X_1^2 - 15.617X_2^2 - 0.052X_3^2 \quad (12)$$

where X_1, X_2, and X_3 are pretreatment time (h), NaOH concentration (%, w/v) and temperature (°C), respectively. *Lignin removal* is the lignin removal rate (%), *Glucose*, *Xylose*, and *Total sugar* are the yields of glucose, xylose, and total sugars (%), respectively. Positive values of the regression coefficient for the above equations suggested a synergistic effect, while negative values indicated an antagonistic effect (Kim *et al*. 2014).

Table 4. ANOVA for the Quadratic Models

Source	Sum of Squares	Degree of Freedom	*F*-value	*P*-value	
Lignin removal					
Model	5264.46	9	250.49	< 0.0001	Significant
X_1	1032.62	1	442.21	< 0.0001	
X_2	2486.89	1	1064.99	< 0.0001	
X_3	987.46	1	422.87	< 0.0001	
$X_1 X_2$	45.09	1	19.31	0.0032	
$X_1 X_3$	0.96	1	0.41	0.5417	
$X_2 X_3$	0.28	1	0.12	0.7389	
Residual	16.35	7			
Lack of fit	9.50	3	1.85	0.2785	Not significant
R^2	0.9969				
Adjusted R^2	0.9929				
Predicted R^2	0.9692				
Glucose					
Model	4876.66	9	125.64	< 0.0001	Significant
X_1	525.37	1	121.82	< 0.0001	
X_2	3029.14	1	702.37	< 0.0001	
X_3	581.40	1	134.81	< 0.0001	
$X_1 X_2$	102.31	1	23.72	0.0018	
$X_1 X_3$	0.66	1	0.15	0.7081	
$X_2 X_3$	31.70	1	7.35	0.0302	
Residual	30.19	7			
Lack of fit	21.62	3	3.37	0.1358	Not significant
R^2	0.9938				
Adjusted R^2	0.9859				
Predicted R^2	0.9268				
Xylose					
Model	1610.76	9	95.23	< 0.0001	Significant
X_1	41.63	1	22.15	0.0022	
X_2	417.03	1	221.90	< 0.0001	
X_3	244.76	1	130.24	< 0.0001	
$X_1 X_2$	74.48	1	39.63	0.0004	
$X_1 X_3$	47.68	1	25.37	0.0015	
$X_2 X_3$	45.70	1	24.32	0.0017	
Residual	13.16	7			

Lack of fit	7.79	3	1.94	0.2656	Not significant
R^2	0.9919				
Adjusted R^2	0.9815				
Predicted R^2	0.9181				
Total sugar					
Model	3285.05	9	170.61	< 0.0001	Significant
X_1	307.15	1	143.57	< 0.0001	
X_2	1838.00	1	859.11	< 0.0001	
X_3	434.09	1	202.90	< 0.0001	
$X_1 X_2$	16.95	1	7.93	0.0259	
$X_1 X_3$	3.90	1	1.82	0.2190	
$X_2 X_3$	0.71	1	0.33	0.5837	
Residual	14.98	7			
Lack of fit	11.21	3	3.97	0.1082	Not significant
R^2	0.9955				
Adjusted R^2	0.9896				
Predicted R^2	0.9439				

X_1 – Time; X_2 – Alkali loading; X_3 – Temperature

Analysis and Optimization of the NaOH Pretreatment Conditions for Monomeric Sugar Release

The main objective of this study was to assess and optimize the reaction conditions for dilute NaOH pretreatment of sorghum pith and maximize the release of monomeric sugars by subsequent enzymatic hydrolysis. Therefore, the most important responses were considered to be the enzymatic hydrolysis yields of the glucose, xylose, and total sugars. The response of the lignin removal was also studied in this section.

As shown in Fig. 2, when the temperature or time was fixed at their center point, an increase in the alkali loading led to a significant removal of lignin. Based on the ANOVA (Table 4), the time, temperature, and alkali loading were found to be significantly effective on the lignin removal ($p < 0.0001$). Besides, it was found that the alkali loading and time interaction also had a strong influence on the lignin removal. Optimized results show that predicted highest lignin removal (91.5%) was obtained at 40 °C for 19.4 h with 2% NaOH. In the previous studies, a considerable degree of lignin removal (approximately 80%) has also been reported for sweet sorghum bicolor straw (McIntosh and Vancov 2010; Wu *et al*. 2011). However, either a high temperature (121 °C) or NaOH loading (> 1 M) should be applied regardless of the short reaction time to achieve a high saccharification efficiency.

The plotted surfaces in Fig. 3 illustrate the effects of the pretreatment conditions on the enzymatic glucose yield. Separate surfaces were created for time *versus* alkali loading, temperature *versus* time, and alkali loading *versus* temperature. The factors not shown in the surfaces were fixed at their central values. Table 4 shows that all three terms (time, temperature, and alkali loading) were found to significantly ($p < 0.05$) affect the glucose yield.

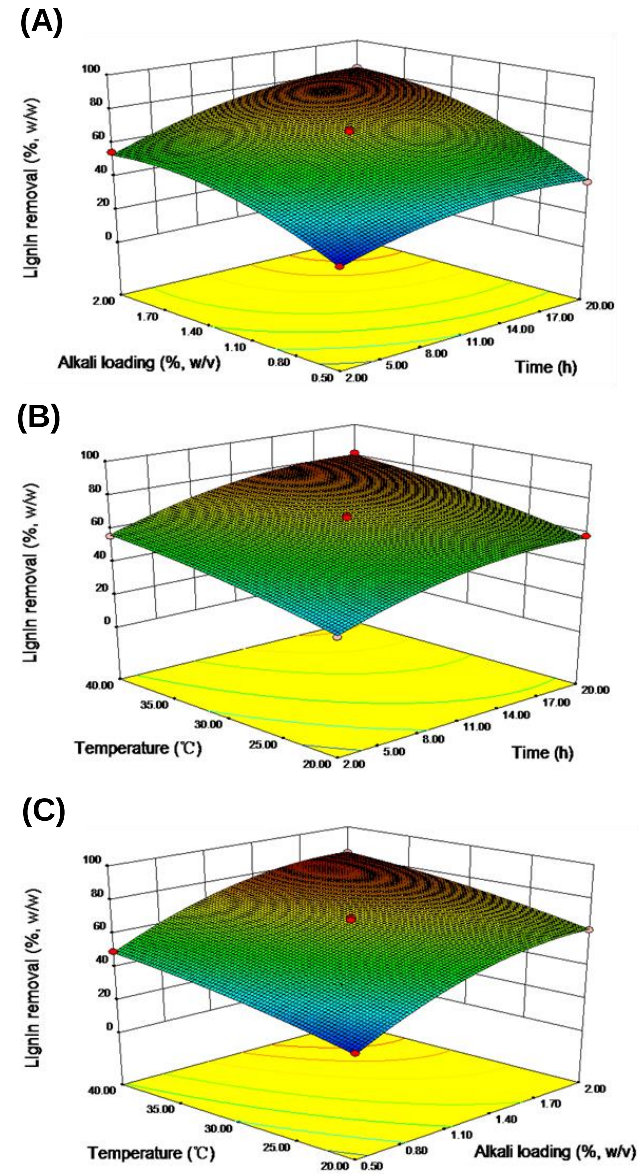

Fig. 2. Surface response plots of the effects of the alkali loading, temperature, and time on the lignin removal: (A) alkali loading *versus* time (fixed temperature at 30 °C); (B) temperature *versus* time (fixed alkali loading at 1.25%); and (C) temperature *versus* alkali loading (fixed time at 11 h)

When the temperature or time was fixed at their center point, an increase in the alkali loading led to a significant increase in the glucose yield (Fig. 3). The glucose yield was most susceptible to the alkali loading compared with the temperature and time, which was determined by the highest F-value of 702.37 (Table 4). This was consistent with the results reported by Wu *et al.* (2011), who also concluded that the alkali concentration had a greater influence on the subsequent enzymatic hydrolysis of sweet sorghum bagasse than the temperature and time. Additionally, it was observed that the glucose yield showed a similar trend as that of the lignin removal rate, which is represented in Fig. 2. This implied that the enzymatic hydrolysis of cellulose is closely related to the lignin removal. This was in agreement with previous studies, which have reported that the enzymatic hydrolysis efficiency increased almost linearly with the lignin removal during alkali pretreatment (Mendes *et al*. 2011; Wu *et al*. 2011). This was because the lignin removal could increase the porosity of cell walls and enhance access of cellulase to the substrate (Chang and Holtzapple 2000; Masarin *et al*. 2011). To maximize the glucose yield (predicted yield of 90.5%), the recommended conditions to treat the sorghum pith are 2% NaOH at 40 °C for 19.5 h.

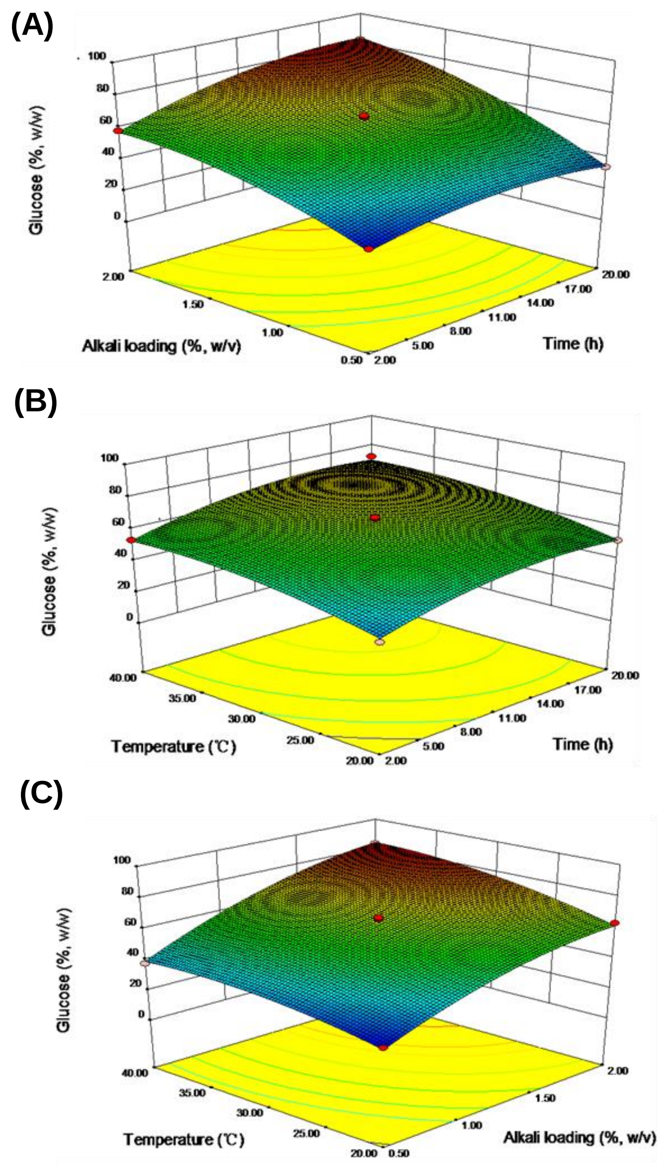

Fig. 3. Surface response plots of the effects of the alkali loading, temperature, and time on the glucose yield: (A) alkali loading *versus* time (fixed temperature at 30 °C); (B) temperature *versus* time (fixed alkali loading at 1.25%); and (C) temperature *versus* alkali loading (fixed time at 11 h)

The effects of the interactions of the alkali loading, temperature, and time on the xylose yield are depicted in Fig. 4. Same as for the glucose yield, the xylose yield was most susceptible to the alkali loading with the F-value of alkali loading higher than that of temperature and time (Table 4). However, unlike the glucose yield, a peak was observed in each response surface plot at the NaOH loading of approximately 1.5%, which indicated that the maximum xylose yield could be obtained within the design boundaries. Figure 4 shows that the xylose yield increased with an increasing alkali loading from 0.5% to 1.4%, reaction time from 2 h to 9.9 h, and temperature from 20 °C to 37.5 °C, but a decline was observed with a further increase in the parameters. The different behaviors between the glucose and xylose yields could be explained by the different loss degrees of glucan and xylan during the NaOH pretreatment. Harsh pretreatment conditions resulted in the excessive loss of xylan from the raw material, which is shown in Fig. 1, and it caused a decrease in the enzymatic xylose yield. In contrast, because the glucan loss from the raw material during the NaOH pretreatment was poor, the enzymatic glucose yield did not exhibit a significantly decreasing trend like the xylose yield did, even if severe pretreatment conditions were applied (Fig. 1).

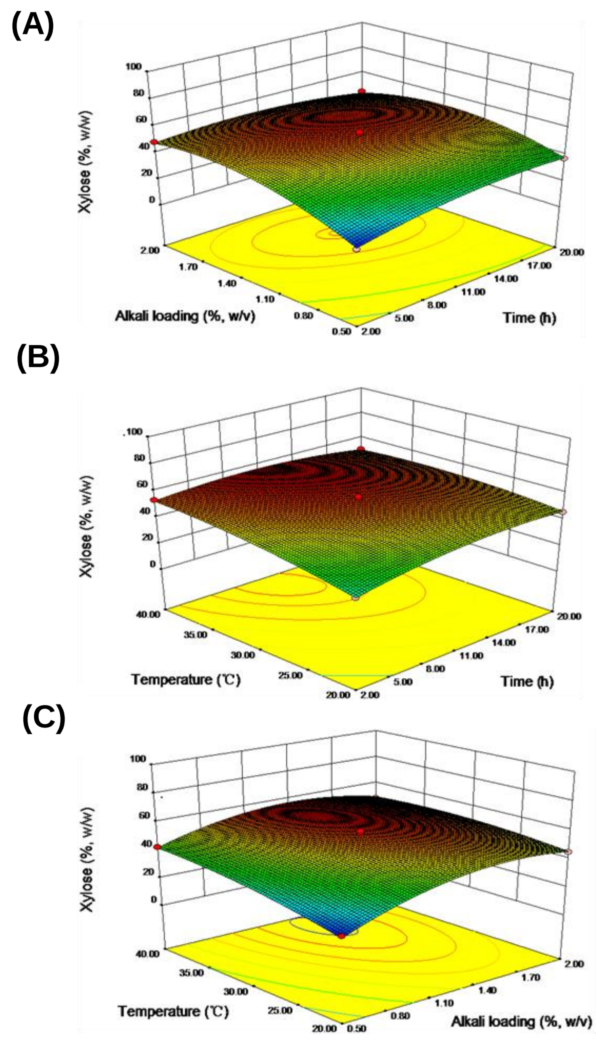

Fig. 4. Surface response plots of the effects of the alkali loading, temperature, and time on the xylose yield: (A) alkali loading *versus* time (fixed temperature at 30 °C); (B) temperature *versus* time (fixed alkali loading at 1.25%); and (C) temperature *versus* alkali loading (fixed time at 11 h)

The optimum conditions for a maximum xylose yield were 9.9 h, 1.4% NaOH, and 37.5 °C, under which the maximum xylose yield was 57.7%. It has been reported in the literature that the optimum pretreatment conditions for maximum glucose and xylose yields were similar to each other during NaOH pretreatment and enzymatic saccharification of sorghum stem (Nikzad *et al*. 2014). However, in this study, the optimum pretreatment conditions for the maximum release of glucose and xylose were significantly different, especially the pretreatment time (19.5 h for glucose and 9.9 h for xylose). This was perhaps caused by the different cell types and cell wall compositions in the sorghum stem and sorghum pith (Hatfield *et al*. 1999). Because the optimum pretreatment conditions for the enzymatic glucose and xylose yields were different, an optimization of the total sugars yield was performed.

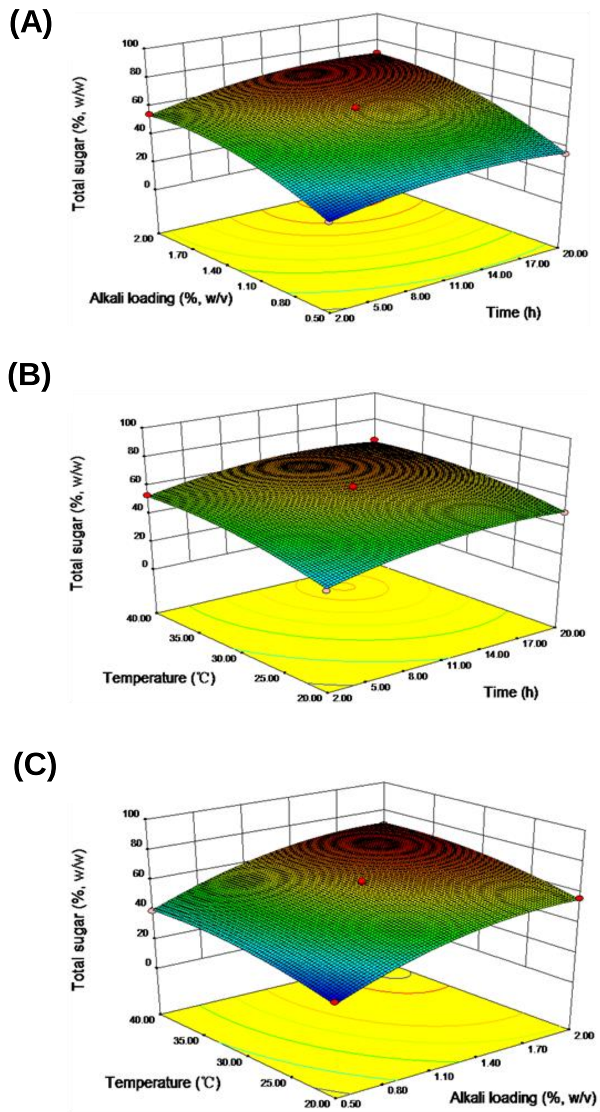

Fig. 5. Surface response plots of the effects of the alkali loading, temperature, and time on the total sugars yield: (A) alkali loading *versus* time (fixed temperature at 30 °C); (B) temperature *versus* time (fixed alkali loading at 1.25%); and (C) temperature *versus* alkali loading (fixed time at 11 h)

The total sugars yield from the pretreated material is demonstrated in Fig. 5. Figure 5A shows the effects of the alkali loading and time on the total sugars yield with the temperature fixed at 30 °C. A significant interaction between the alkali loading and time was observed, which was confirmed by the low p-value (0.0259) from the ANOVA test (Table 4). The interaction of the time and temperature indicated that a maximum sugar yield could be obtained from 14 h to 17 h at 35 °C to 40 °C (Fig. 5B). With an increase in the temperature and time during the pretreatment process, the total sugars yield increased to some extent, but it began to decline with a further increase in these parameters because of the reduced xylose yield. Figure 5C illustrates the effects of the alkali loading and temperature on the total sugars yield. When the time was fixed at 11 h, the alkali loading and temperature were hardly interdependent, which was confirmed by a high *p*-value of 0.5837 (Table 4).

The optimum pretreatment conditions for the maximum total sugars yield were 2% NaOH, 16.2 h, and 37 °C. With these conditions, the predicted total sugars yield was 73.9% after enzymatic hydrolysis, and the glucose and xylose yields were 87.8% and 47.4%, respectively. With these optimum conditions, experiments were conducted to confirm the predicted values, which showed a glucose yield of 87.2%, xylose yield of 48.2%, and total sugars yield of 72.4%. The good agreement of the experimental values with the predicted values indicated the validity and adequacy of the models. To date, several researchers have studied the NaOH pretreatment of sorghum stem (McIntosh and Vancov 2010; Wu *et al.* 2011; Nikzad *et al.* 2014). However, either a high NaOH dosage or reaction temperature was applied despite a considerable enzymatic glucose yield in these studies. To the knowledge of the authors, this study is the first report on optimizing the conditions of dilute NaOH pretreatment of sorghum pith at a mild temperature for a maximum total monomeric sugars yield by enzymatic hydrolysis.

Validation of the Developed Models

In order to verify the predicted capacity of the models, further experiments were carried out under the optimized conditions. The results for lignin removal, glucose, xylose and total sugar were respectively compared with the predicted values by the developed models. As shown in Table 5, all the experimental values are very close to the predicted values, which indicate the validity and adequacy of these models.

Table 5. Confirmation of the Predicted Optimum Condition with the Experimental Values

	Temperature (°C)	Time (h)	NaOH loading (%)	Predicted value (%)	Actual value (%)
Lignin removal	40	19.4	2	91.5	92.2 ± 0.8
Glucose yield	40	19.5	2	90.5	90.7 ± 0.7
Xylose yield	37.5	9.9	1.4	57.7	56.9 ± 0.8
Total sugar yield	37	16.2	2	73.9	72.4 ± 1.6

Effect of the Enzyme Loading on the Enzymatic Hydrolysis of the Pretreated Sorghum Pith

The pretreated material (obtained at 37 °C for 16 h with 2% NaOH) was further processed by enzymatic hydrolysis with different enzyme loadings to optimize the enzyme dosage. Here, the glucose and xylose yields were calculated on the basis of the glucan and xylan in the pretreated material, respectively.

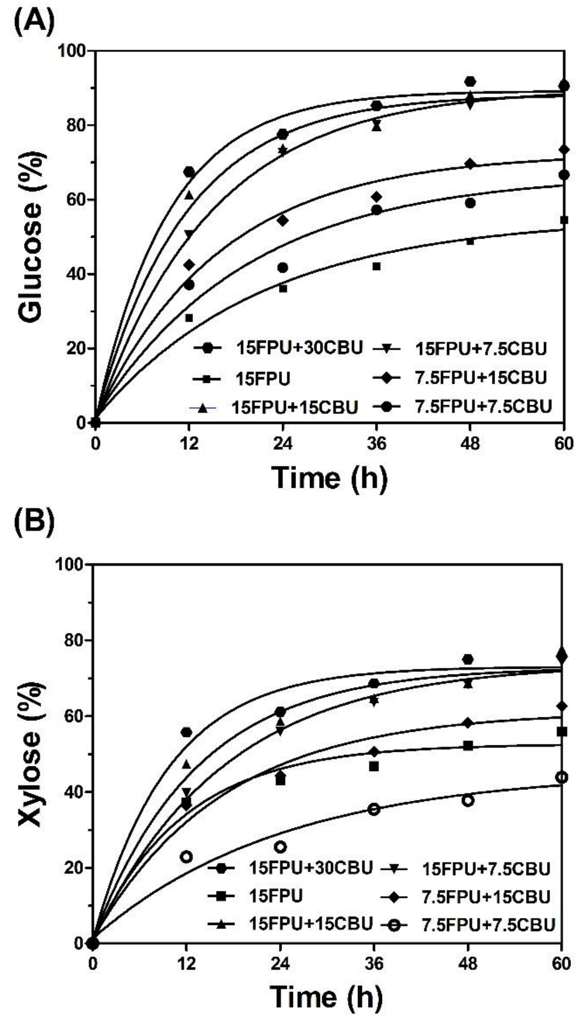

Fig. 6. Effect of the enzyme dosage on the glucose (A) and xylose (B) yields from the pretreated sorghum pith (2% NaOH, 16 h, and 37 °C) after enzymatic saccharification (5%, w/v; 50 °C; pH = 4.8)

The enzymatic glucose and xylose yields were only 54.6% and 56%, respectively, when 15 FPU cellulase/g of pretreated material was used. After the addition 7.5 CBU *β*-glucosidase/g of pretreated material to 15 FPU cellulase/g of pretreated material, the 60-h enzymatic hydrolysis glucose yield clearly increased to 90.2%, while the xylose yield increased to 74.3%. This indicated that the degradation of cellulose could promote the hydrolysis of xylan, which agreed well with the conclusions of Zhang and Viikari (2014), who stated that cellulase could synergistically increase the accessibility of xylanase to xylan because xylan was also partially covered by layers of cellulose. In contrast, the enzymatic glucose and xylose yields did not significantly increase when the *β*-glucosidase dosage further increased to 15 CBU/g of pretreated material and 30 CBU/g of pretreated material, which suggested that the enzymatic loading saturation point might have been reached. Also, when the addition of cellulase/*β*-glucosidase was 7.5 FPU/7.5 CBU and 7.5 FPU/15 CBU per gram of pretreated material, the glucose and xylose yields from the 60-h enzymatic hydrolysis were lower than those with the addition of 15 FPU/7.5 CBU per gram of pretreated material. Therefore, the addition of 15 FPU/7.5 CBU per gram of pretreated material for 60-h enzymatic hydrolysis is optimal for the release of glucose and xylose from alkali-pretreated sorghum pith.

SEM and XRD Analysis of the Raw and Pretreated Sorghum Pith

The SEM images of the raw and pretreated materials (37 °C, 2% NaOH, 16 h) are displayed in Figs. 7A and 7B, respectively. The raw material had a compact and inaccessible structure with a smooth surface morphology. In contrast, the surface was significantly damaged after the NaOH pretreatment. The NaOH pretreatment deconstructed the lignocellulose matrix and made the surfaces of the sorghum pith swell because of the partial removal of hemicellulose and dissolution of lignin (Cabrera *et al*. 2015). The bioaccessibility was ultimately enhanced by the increased surface areas and disrupted lignocellulosic structure of the sorghum pith.

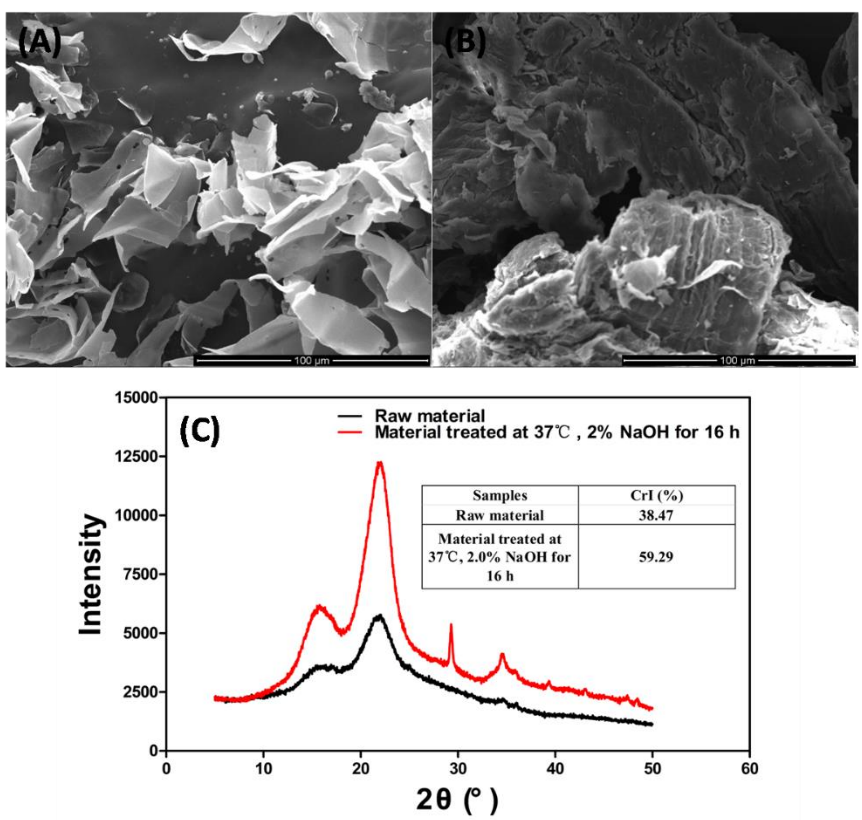

Fig. 7. (A) SEM image of the raw sorghum pith under 800x magnification; (B) SEM image of the NaOH-pretreated sorghum pith (37 °C, 2% NaOH, and 16 h) under 800x magnification; and (C) X-ray diffractogram of the raw and NaOH-pretreated sorghum pith (37 °C, 2% NaOH, and 16 h)

Figure 7C compares the XRD profiles (diffraction intensity *versus* 2θ) of the raw and pretreated materials (37 °C, 2% NaOH, and 16 h). The *CrI* of the pretreated material was found to increase significantly from 38.47 to 59.29. This was because the amorphous components of the hemicellulose and lignin were remarkably removed after the NaOH pretreatment and the crystalline cellulose content in the pretreated material increased compared with that in the raw material (He *et al*. 2015; Li *et al*. 2016).

FT-IR Spectrometric Analysis of the Raw and Pretreated Sorghum Pith

The chemical groups of sorghum pith before and after NaOH pretreatment (37℃, 2% NaOH and 16 h) are displayed in Fig. 8. A shoulder at 1737.5 cm^{-1} was attributed to the ester-linked acetyl, feruloyl, and *p*-coumaroyl groups on hemicelluloses and/or lignin (Gabhane *et al.* 2015). The bands at 1737.5 cm^{-1} of the pretreated material completely disappeared, suggesting that the pretreatment nearly cleaved these ester bands from the hemicelluloses and lignin. The characteristic bands of lignin could be found at 1255.4, 1515.8, and 1608.3 cm^{-1}, and they are assigned to phenolic C-O, aromatic ring stretch vibrations and aromatic skeletal vibrations plus C=O stretching, respectively (Wang *et al.* 2015). The disappearence of the three lignin-associated peaks demonstrated the removal of the lignin after NaOH pretreatment. The carbohydrate-related peaks at 1390.4, 1168.6, and 1058.7 cm^{-1} were respectively referred to the C-H deformation, C-O-C vibration and C-O vibration in both cellulose and hemicelluloses (He *et al.* 2015). The decrease in these absorption bands of pretreated material is an indication of decrease in these linkages as a result of removal of hemicelluloses and cellulose associated with each other and with lignin. The absorption at 902.5 cm^{-1} was the characteristic of β-glycosidic linkages and its relative absorption decreased significantly after NaOH pretreatment (Ma *et al.* 2015). This implied that the β-glycosidic linkages were disrupted and carbohydrates depolymerized after alkali pretreatment. Based on the above data, it can be found that the structure of the pretreated sorghum pith was obviously destroyed and alkali pretreatment leaded to significant change of chemical groups.

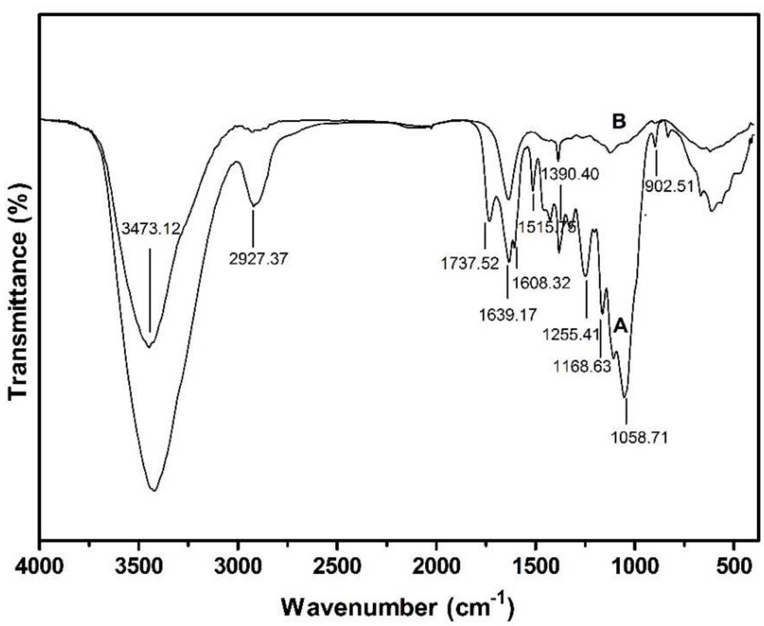

Fig. 8. FT-IR spectra of raw material (A) and NaOH pretreated material at 37℃, 2.0% NaOH for 16 h (B)

CONCLUSIONS

1. Dilute alkali pretreatment at low temperatures resulted in the partial removal of xylan and lignin from the sorghum pith, whereas most of the glucan remained in the pretreated material.
2. The pretreatment conditions for the maximum fermentable monomeric sugars yield from enzymatic hydrolysis were 37 °C, 16 h, and 2% NaOH. Under these conditions, the yield of total monomeric sugars (based on the glucan and xylan in the raw material) was 72.4%, which included a glucose yield of 87.2% and xylose yield of 48.2%.
3. The enzymatic glucose and xylose yields of the pretreated sorghum pith (37 °C, 16 h, and 2% NaOH) were 90% and 74%, respectively (based on the glucan and xylan in the pretreated material), when using 15 FPU cellulase and 7.5 CBU β-glucosidase per gram of pretreated material. This implied that a diluted NaOH pretreatment at a low temperature could be successfully applied to sorghum pith to efficiently release monomeric sugars while operating at low enzyme charges.

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