Lime pretreatment of maize stover and solubilization of pretreated solids by enzymatic hydrolysis and *Clostridium thermocellum* fermentation

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Abstract: Lime pretreatment on maize stover was conducted with various pretreatment conditions selected by central composite design. Xylan and lignin contents in pretreated maize stover were relatively constant. Concentration of glucose monomer and oligomers decreased while concentration of xylooligomers increased in the pretreatment hydrolysate with increasing pretreatment intensity. The overall carbohydrate recovery was at least 85% for the conditions studied. Xylan removal during pretreatment was found to have a linear correlation with lignin removal. Pretreatment had a higher effect in enhancing carbohydrate solubilization for enzymatic hydrolysis than that for *C. thermocellum* fermentation. For all the pretreated solids, *Clostridium thermocellum* fermentation was found to result in much higher carbohydrate solubilization than enzymatic hydrolysis with a cellulase loading of 8 mg/g solids and a xylanase loading of 2 mg/g solids. Carbohydrate solubilization was found to have a linear correlation with lignin removal during lime pretreatment for both enzymatic hydrolysis and *C. thermocellum* fermentation. Considering the current challenges, this research provides a new idea for the industrial application of lignocellulosic biorefinery.

Keywords: central composite design, lime pretreatment, enzymatic hydrolysis, *clostridium thermocellum*, carbohydrate solubilization

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1 Introduction

Lignocellulosic biomass is of interest as a sustainable source of organic fuels, chemicals, and materials because of its large scale potential availability, low purchase cost, and more desirable environmental attributes as compared to row crops[1]. Of particular interest is the abundant agricultural waste such as maize stover. However, lignocellulosic biomass is recalcitrant to attack by cellulase enzymes and microorganisms due to its complex structure[2]. Pretreatment is typically required to open its structure for enzymatic and microbial conversion. Fungal cellulase is commonly used to hydrolyze carbohydrates in lignocellulosic biomass. *Clostridium thermocellum* is a widely studied microorganism due to its ability to rapidly hydrolyze lignocellulosic material and ferment the hydrolysis products to ethanol accompanied by organic acids[3,4].

The recalcitrance of lignocellulosic biomass can be attributed to a number of factors such as lignin-cellulose complex, cellulose polymerization degree, cellulose crystallinity, lignin content, and lignin structure and distribution[5,6]. Lignin-related factors, such as lignin content, lignin distribution and physical structure, the connection between lignin and carbohydrates, and the hydrophobic effect between lignin and enzymes, are considered to be important influencing factors[7,8]. It is generally believed that lignin can reduce the efficiency of enzyme action through non-specific binding of cellulase to lignin and lignin acting as a physical barrier to the accessibility of cellulose to enzymes[8,9]. Removal of lignin usually enhances the enzymatic hydrolysis efficiency of substrates[10,11].

Various pretreatment technologies have been studied to make lignocellulosic biomass more amenable to enzyme and microbial conversion[12-15]. Alkaline pretreatment is considered as one of the most effective methods due to effective delignification, minimal interaction with hemicellulose, and less toxic byproduct production[16,17]. The effectiveness of alkaline pretreatment depends on structure and composition of substrates and pretreatment conditions such as alkaline loading, temperature, and time. The main reactions in alkaline pretreatment include deesterization of intermolecular ester bonds and dissolution of lignin and hemicellulose[18].

Cellulase is enzymes produced by microorganisms that can degrade natural cellulose. It has attracted worldwide attention and its research has made great progress[19-22]. Although a variety of microorganisms have been found to produce cellulase, commercial cellulase is mostly produced by *Trichoderma reesei*. Commercial cellulases have improved significantly over the past several decades. However, large scale commercial application of cellulase for lignocellulosic biomass conversion still has obstacles due to high cellulase cost and relatively large quantity needed for good hydrolysis performance[23,24]. The breeding of highly efficient cellulase producing biological groups is still one of the focusing areas in cellulase research[25,26].

Clostridium thermocellum is a candidate microorganism for consolidated bioprocessing (CBP). The mechanism of cellulose solubilization by *C. thermocellum* is different from that of fungal cellulase[3]. *C. thermocellum* produces a supramolecular multi-enzyme complex comprised of a wide variety of polysaccharide-

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degrading enzymes and is the most efficient microorganism for solubilizing lignocellulosic biomass known to date[27,28]. Previous studies have reported that *C. thermocellum* fermentation could efficiently solubilize lignocellulosic biomass with minimal pretreatment[29,30].

Of various reagents used for alkaline pretreatment, lime or $Ca(OH)_2$ is of great interest due to its low cost. Lime pretreatment on maize stover was examined in this study. Carbohydrate solubilization by fungal cellulase hydrolysis and *C. thermocellum* fermentation was studied for lime pretreated maize stover. Performance on carbohydrate solubilization was compared for the two systems to give insights on how the two systems react to the same pretreatment conditions.

2 Materials and methods

2.1 Feedstock and its preparation

The feedstock maize stover was kindly provided by Qing's lab at the Changzhou University, Jiangsu Province, China. The maize stover was milled to pass through a sieve mesh size of 30 using a hammer mill (YF8-1, Yongli, Wenzhou, China) and then dried in an oven (GFL-125, Labotery, Tianjin, China) at 60°C for 24 h. Dry maize stover was stored in glass flasks at room temperature for subsequent experiments. The raw maize stover contained 35.37% glucan, 19.00% xylan, and 15.47% lignin with drying at 60°C for 24h. The maize stover had a 25.45% fraction that was water-soluble, which contained 13.01% glucose monosaccharide, 0.94% glucose oligomers and 6.67% xylose oligomers relative to total carbohydrates in the raw maize stover.

2.2 Enzymes

Cellulase and xylanase samples were both provided by the Vland Biotechnology Co., LTD (Qingdao, Shandong, China). The cellulase sample contained 63 mg/mL protein with an activity of 118 FPU/mL and the xylanase sample contained 2 mg/mL protein with an activity of 8000 U/mL for beechwood xylan. Enzymes were stored at 4°C.

2.3 Strains and medium

Clostridium thermocellum DSM 1313 was used in this study. Chemically-defined media for *thermophilic clostridia* (MTC), with components in solutions A (MOPS buffer without carbohydrate), B (citrate and bicarbonate buffer), C (nitrogen source), D (minerals and reducing agent), and E (vitamins), was prepared according to Shao et al.[11].

2.4 Experimental design and statistical analysis

In this study, three factors including lime loading, pretreatment temperature and pretreatment time were chosen as critical variables. The central composite design was used to reduce the number of experiments required to determine the relationship between composition changes of pretreated maize stover and their glucan or xylan solubilization. The statistical software Design Expert 10 (Stat-Ease, MN, USA) was used for the 3×3 central composite design in which 16 pretreatment combinations were derived. Factor levels for lime loading, pretreatment temperature and pretreatment time were selected based on previous studies[31,32]. The range of lime loading ($Ca(OH)_2$/dry maize stover) was between 0.01 and 0.15 g/g, pretreatment temperature was between 75°C and 125°C, and pretreatment time was between 30-240 min. A list of pretreatment conditions generated by the software was shown in Table 1.

After data were collected according to the experimental design, an f-test was performed to check the significance of data. The degree of fitting to the polynomial model equation was represented by the determination coefficient (R^2). Unless otherwise noted, all statistical analyses were performed in Design Expert 10. If the *p*-value of the model was less than 0.05, it was considered to be significant.

Table 1 Central composite design of lime pretreatment of maize stover

Std sequence[a]	Run sequence[b]	Lime loading [$Ca(OH)_2$/dry maize stover]/(g·g^{-1})	Pretreatment Temp/°C	Pretreatment Time/min
9	1	0.01	100	135
3	2	0.04	115	73
7	3	0.04	115	197
10	4	0.15	100	135
12	5	0.08	125	135
5	6	0.04	85	197
15[c]	7	0.08	100	135
2	8	0.12	85	73
13	9	0.08	100	30
8	10	0.12	115	197
14	11	0.08	100	240
6	12	0.12	85	197
4	13	0.12	115	73
16[c]	14	0.08	100	135
11	15	0.08	75	135
1	16	0.04	85	73

Note: [a] Standard sequence was designed by Design-Expert 10 and was fixed. [b] Run sequence was actual experiment sequence and was random. [c] Center point of the central composite design.

2.5 Lime pretreatment

An appropriate amount of calcium hydroxide was added to 2 g of maize stover in 100-mL serum bottles. After adding 20 mL ultrapure water, the bottles were plugged with butyl rubber stoppers, sealed with aluminum caps, and then shaken to mix the contents evenly. Lime pretreatment was conducted in an autoclave (LX-B50, Huatai Medical, Hefei, China). The bottles were taken out of the autoclave after pretreatment when temperature was 100°C or below. After cooling to room temperature, pretreatment hydrolysate and residual solids were collected. All pretreatments were conducted in triplicates unless otherwise stated.

2.6 Enzymatic hydrolysis

Enzymatic hydrolysis was performed in 100-mL serum bottles. 0.25 g pretreated maize stover (dried at 60°C) was added into serum bottles supplemented with 47.22 mL ultrapure water. The bottles were sterilized by autoclaving at 121°C for 30 min to be consistent with the preparation of pretreated maize stover for *C. thermocellum* fermentation. After cooling to room temperature, 2.5 mL, 1 mol/L citrate buffer (pH 5.0) supplemented with 50 mg/L kanamycin (20X) was added to the bottles. 0.032 mL cellulase and 0.25 mL xylanase were added to the bottles, resulting in a typical total enzyme loading of 10 mg/g (protein/dry maize stover) with a ratio of 4:1 for cellulase compared to xylanase. The total reaction volume was 50 mL with a concentration of 5 g/L pretreated solids. The bottles were incubated in a shaking incubator with rotation speed set at 200 r/min and temperature controlled at 50°C for 72 h. Unless otherwise noted, all enzymatic hydrolysis experiments were carried out in three replicates. After enzymatic hydrolysis, 2 mL hydrolysate was transferred into a 2-mL centrifuge tube and centrifuged at 12 000 r/min for 1 min (D3024, DLAB, Beijing, China). The supernatant was filtered with a 0.22-micron nylon filter (25 mm, Jin Teng, Tianjin, China). 0.005 mL 72% (wt) sulfuric acid and 1.495 mL ultrapure water were added to 0.5 mL filtrate to

acidify and dilute the sample, which was then measured for glucose and xylose concentrations by high performance liquid chromatography (Shimadzu, Japan).

2.7 *Clostridium thermocellum* fermentation

C. thermocellum fermentation was performed in 100-mL serum bottles. 0.25 g pretreated maize stover (dried at 60°C) was added into serum bottles supplemented with 39 mL ultrapure water. The bottles were plugged with butyl rubber stoppers and sealed with aluminum caps. After purging with ultrapure N2, the bottles were sterilized by autoclaving at 121°C for 30 min. After cooling to room temperature, 5mL A, 2 mL B, 1 mL C, 1 mL D, 1 mL E, and 1 mL *C. thermocellum* inoculum prepared in MTC medium with 5 g/L Avicel at 50°C for 24h, were injected into the bottles with sterile syringes and needles. The bottles were incubated in a shaking incubator with rotation speed set at 200 r/min and temperature controlled at 50°C for 72 h. After *C. thermocellum* fermentation, the reaction contents were transferred into a 50-mL centrifuge tube that had been weighed in advance, followed by washing the remaining solids three times by centrifuging, removing supernatant and re-suspending with water to sampling volume. The wet residual solids were then dried at 60°C to constant weights to determine the amount of remaining solids. Samples of dry residual solids were taken for composition analysis to determine glucan and xylan contents, which were used to calculate their solubilization by *C. thermocellum* fermentation. Unless otherwise noted, all *C. thermocellum* fermentations were carried out in three replicates.

2.8 Analytical methods

The water soluble fraction of maize stover was determined by measuring weight of remaining solids after incubating 1g sample in 50 mL water in 100-mL serum bottles at 50°C in a shaking incubator for 1 h, followed by washing the remaining solids three times by centrifuging, removing supernatant and re-suspending with water to sampling volume. The pretreatment hydrolysate was recovered by centrifuging at 12 000 r/min for 1 min and the supernatant was filtrated with a 0.22-micron nylon filter. For carbohydrates in the water soluble fraction of maize stover and pretreatment hydrolysates, dilute acid hydrolysis was performed by adding 1 mL 72% (wt) H_2SO_4 to 28 mL supernatant and autoclaving at 121°C for 1 h. The residual solids after lime pretreatment were neutralized with HCl and were collected by centrifuging at 12 000 r/min for 5 min (Neofuge 18R, Heal Force, Shanghai, China). The solids were washed five times by removing the supernatant, re-suspending to the sampling volume, and centrifuging again. The wet residual solids were then dried at 60°C to constant weights to determine the amount of remaining solids and percent of solids recovery. Carbohydrate contents for solids were analyzed using the standard laboratory analytical procedures developed by the National Renewable Energy Laboratory (NREL). Concentrations of glucose and xylose were measured using a Shimadzu LC series modular HPLC equipped with an Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) operated at 60°C and a refractive index detector. The mobile phase was 5 mmol/L H_2SO_4 at a flow rate of 0.5 mL/min.

The solid recovery, total carbohydrate recovery, lignin removal, enzymatic hydrolysis solubilization, and *C. thermocellum* fermentation solubilization were calculated as follows:

$$a = \frac{b}{c} \times 100\% \quad (1)$$

where, a=solid recovery; b=pretreated corn stover, g; c=raw corn stover, g.

$$d = \frac{e+f}{g} \times 100\% \quad (2)$$

where, d=total carbohydrate recovery; e=sugars in pretreated corn stover, g; f=sugars in pretreated hydrolysate, g; g=sugars in raw corn stoverg.

$$h = \frac{i-j}{i} \times 100\% \quad (3)$$

where, h=lignin removal; i=lignin in raw corn stover, g; j=lignin in pretreated corn stover, g.

$$k = \frac{l}{e} \times 100\% \quad (4)$$

where, k=solubilization of carbohydrate by enzymatic hydrolysis; l=sugars in enzymatic hydrolysate, g;

$$m = \frac{e-n}{e} \times 100\% \quad (5)$$

where, m=solubilization of carbohydrate by *C. thermocellum* fermentation, n=sugars in residual corn stover, g.

3 Results and discussion

3.1 Composition analysis of lime pretreated maize stover and carbohydrates in the pretreatment hydrolysate

Composition analysis of pretreated maize stover obtained from 16 pretreatment tests derived from the central composite design using the Design-Expert software was listed in Table 2.

The data were arranged from low to high value in the order of lime loading, pretreatment temperature and pretreatment duration (might not be in line with pretreatment severity). Data in Table 3 and Table A2 were also arranged in this way. As listed in Table 2, compared to maize stover without lime pretreatment (glucan 35.37%, xylan 19.00% and lignin 15.47%), glucan content of maize stover after lime pretreatment increased by 3.56% to 8.32%, while xylan content increased by 0.65 to 2.96% and lignin content increased by 2.57 to 4.27%. The increase in glucan content was expected for alkaline pretreatment because alkaline pretreatment typically resulted in xylan and lignin removal, which concentrated glucan in the pretreated material[33,34]. However, the contents of xylan and lignin in the lime pretreated maize stover increased in this study. This was because the unpretreated maize stover contained 25.45% water-soluble fraction. When lime pretreatment was in progress, the water-soluble fraction was dissolved in water, which could effectively result in an increase in xylan and lignin contents in the remaining water-insoluble solids. The xylan and lignin contents for lime pretreated solids under various pretreatment conditions remained relatively constant. This was because lime pretreatment was much less harsh than sodium hydroxide pretreatment for xylan and lignin removal[35], and intensification of pretreatment conditions was accompanied by loss of more insoluble solid (Table 3).

In the hydrolysate solution of lime pretreatment, there were mainly three carbohydrate components including glucose monosaccharide, glucose oligosaccharide and xylose oligosaccharide. No xylose monosaccharide was detected in the hydrolysate solution, indicating that no xylose was produced or xylose was degraded in the lime pretreatment process. With the intensification of pretreatment conditions, the content of glucose monosaccharide and glucose total carbohydrate (monosaccharide and oligosaccharide) in the hydrolysate solution decreased gradually (glucose from 5.46 to 1.47 g/L, glucose total carbohydrate from 7.08 to 2.48 g/L), indicating that degradation of glucose occurred in the lime pretreatment process and the more intense the pretreatment

conditions, the higher the degradation of glucose. However, the change of glucose oligosaccharide content (from 1.62 to 1.01 g/L) in the pretreatment hydrolysate was much less than that of glucose monosaccharide. This was consistent with previous studies that xylose and glucose monomers were more susceptible to degradation in alkaline conditions[36,37]. Xylooligomer concentration in the pretreatment hydrolysate generally increased with increasing pretreatment intensity (from 1.99 to 5.46 g/L). This was caused by solubilization of xylan by alkaline[38-40].

Table 2 Carbohydrate concentration in pretreatment hydrolysate and composition of pretreated maize stover

Std Sequence	Lime loading [$Ca(OH)_2$/dry maize stover]/(g·g^{-1})	Pretreatment Temp/°C	Pretreatment Time/min	Pretreatment hydrolysate (V=20 mL)			Pretreated solids[b]		
				Monosaccharide Glucose/g·L^{-1}	Total carbohydrate[a] Glucose/g·L^{-1}	Total carbohydrate Xylose/g·L^{-1}	Glucan/%	Xylan/%	Lignin/%
9	0.01	100	135	5.464	7.083	1.989	38.96	21.81	19.53
1	0.04	85	73	5.061	6.517	2.109	39.46[d]	21.53[d]	19.50[d]
5	0.04	85	197	5.025	6.326	2.319	40.13	21.79	19.47
3	0.04	115	73	4.372	5.807	2.878	39.92	21.92	19.70
7	0.04	115	197	4.037	5.519	2.977	40.14	21.96	19.77
11	0.08	75	135	3.967	5.076	3.127	40.40	21.42	19.53
13	0.08	100	30	3.125	4.158	3.409	41.95	21.87	19.67
15[c]	0.08	100	135	2.633	3.724	3.820	42.61	21.36	19.57
16[c]	0.08	100	135	2.663	3.696	3.843	42.87	21.49	19.67
14	0.08	100	240	2.564	3.816	4.012	41.48[d]	21.08[d]	19.30[d]
12	0.08	125	135	1.962[d]	2.850	4.363	43.01	21.42	18.53
2	0.12	85	73	2.112	3.269	3.874	42.57	21.70	18.83
6	0.12	85	197	1.696	2.791	4.516	43.41	21.23	18.37
4	0.12	115	73	1.507	2.770	5.108	43.10	20.35	18.33
8	0.12	115	197	1.496	2.690	5.462	43.72	19.65	18.07
10	0.15	100	135	1.465	2.480	4.201	42.76	21.27	18.60

Note: [a] Total carbohydrate was monosaccharide-equivalent including monosaccharides and oligosaccharides (no monosaccharide for xylose). [b] The composition of pretreated solids was based on oven-dry weight. [c] Center point of the central composite design. Data were mean values of three replicates ([d] mean values of two replicates).

Table 3 Recovery of solids and carbohydrate and removal of xylan and lignin for lime pretreatment of maize stover

Std sequence	Lime loading [$Ca(OH)_2$/dry maize stover]/(g·g^{-1})	Pretreatment Temp/°C	Pretreatment Time/min	Solid Recovery/%	Total carbohydrate recovery/%[a]		Removal/%[b]	
					Glucan	Xylan	Xylan	Lignin
9	0.01	100	135	72.43	97.72	92.36	16.85	8.72
1	0.04	85	73	71.75	96.55[d]	91.07[d]	18.69	9.73
5	0.04	85	197	70.73	96.27	91.85	18.89	11.15
3	0.04	115	73	70.20	93.93	94.31	19.02	10.78
7	0.04	115	197	68.94	92.20	93.46	20.33	12.09
11	0.08	75	135	69.72	92.47	93.09	21.39	12.15
13	0.08	100	30	68.99	92.33	95.20	20.59	12.46
15[c]	0.08	100	135	67.71	90.96	93.81	23.88	14.51
16[c]	0.08	100	135	67.72	91.42	94.39	23.41	14.06
14	0.08	100	240	68.08	89.47[d]	94.11[d]	24.47	15.23
12	0.08	125	135	66.43	87.96	95.10	25.11	20.58
2	0.12	85	73	68.68	90.90	96.39	21.56	16.56
6	0.12	85	197	66.34	88.44	95.05	25.87	21.38
4	0.12	115	73	64.31	85.34	92.52	31.14	23.95
8	0.12	115	197	63.41	85.14	90.89	34.41	26.08
10	0.15	100	135	65.56	85.49	92.84	26.62	21.33

Note: [a] Total carbohydrate recovery was calculated based on the initial carbohydrate in raw maize stover and end carbohydrate in the pretreatment hydrolysate and pretreated solids. [b] Removal (%) was calculated based on xylan and lignin in the pretreated solids and raw maize stover. [c] Center point of the central composite design. Data were mean values of three replicates ([d] mean values of two replicates).

3.2 Recovery of solids and total carbohydrates

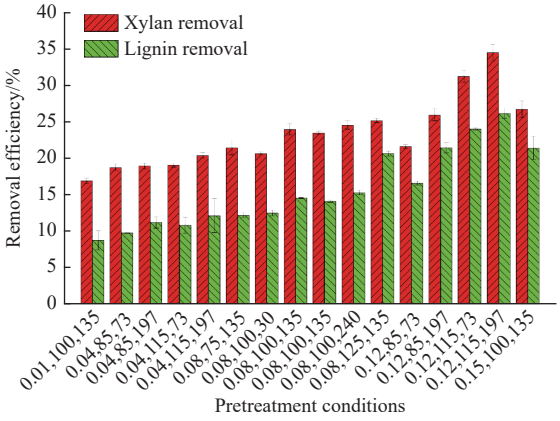
The recovery of solids and the recovery of total carbohydrates (both in pretreatment hydrolysate and in residual solids) for lime pretreatment of maize stover were listed in Table 3. The solids recovery for the selected pretreatment conditions was between 63.41% and 72.43%. The experimental group with the lowest intensity of pretreatment (0.01 g $Ca(OH)_2$/g dry maize stover, 100°C and 135 min) resulted in the highest solids recovery (72.43%), which was slightly lower than the water-insoluble solids content in the unpretreated maize stover (74.55%). This indicated that minimal amount of solids was removed due to lime pretreatment (<3%) under those conditions. With the intensification of lime pretreatment conditions, the solids recovery decreased gradually for the overall trend with the exception for the condition with the maximum concentration of calcium hydroxide 15% (w/w), which resulted in a solids recovery of 65.56%. Although the alkali concentration was one of the most important factors, the pretreatment effect was the result of a combination of lime concentration, time and temperature[41,42].

As listed in Table 3, glucan recovery was between 85.14% and

97.72%. With the intensification of pretreatment conditions, glucan recovery decreased gradually. The presence of xylose monosaccharide was not detected in the pretreatment hydrolysate and in the water soluble fraction of maize stover, indicating that xylose was either not produced or degraded completely during pretreatment. Xylan recovery was between 90.89% and 96.39%. Lime pretreatment of maize stover resulted in the loss of both glucan and xylan. Alkaline pretreatment typically resulted in degradation of carbohydrates[43,44]. Both monomers and oligomers were reported to be degraded directly during pretreatment[39,45,46].

3.3 Removal of xylan and lignin

Removal of xylan and lignin during lime pretreatment of maize stover was given in Table 3 and their trends with various pretreatment conditions were shown Figure 1.



Error bars were standard deviations; The numbers on the horizontal axis were for lime loading (% wt), temperature (°C), and time (min). (Same as below).

Figure 1 Removal of xylan and lignin under various pretreatment conditions

Removal of xylan was between 16.85% and 34.41%, and removal of lignin was between 8.72% and 26.08%. The extend of xylan and lignin removal in maize stover by lime pretreatment was lower than that of other alkaline pretreatments, which may be due to the low solubility of $Ca(OH)_2$ itself and the crosslinking of calcium ions with lignin during the pretreatment process, thus preventing further degradation of lignin[47]. As shown in Figure 1, removal of xylan and removal of lignin were quite parallel for various pretreatment conditions. Removal of xylan and removal of lignin were both at the minimum when pretreatment conditions were 0.01 g/g [$Ca(OH)_2$/dry maize stover], 100°C and 135 min. When the pretreatment conditions were 0.12 g/g [$Ca(OH)_2$/dry maize stover], 115°C and 197 min, removal of xylan and removal of lignin were both at the maximum. This could be that xylan and lignin have groups that react with alkali. The acetyl groups on xylan were removed under alkaline conditions, and the ether bonds on lignin were attacked under alkaline conditions[13,48]. Xylan and glucan were mainly connected by non-covalent bonds such as hydrogen bond and van der Waals force, while xylan and lignin were linked by covalent bonds[49]. Thus, xylan and lignin were more likely to break off from the lignin-carbohydrate complex together during pretreatment.

Statistical analysis for the effect of lime pretreatment on xylan and lignin removal of maize stover was listed in Table A1. Xylan and lignin removal were fitted by quadratic polynomial regression equations. When fitting the xylan removal, the model's determination coefficient R^2 was 0.93. The p value of the model was 0.0075, less than the critical value p=0.05, indicating that the model was significant. Among the three variables (two first-order effects and one interaction effect) that had significant effects on xylan removal of maize stover, the effect of calcium hydroxide loading was the most significant (p=0.0003, regression coefficient was 3.93). When fitting the lignin removal, the model's determination coefficient R^2 was 0.955. The p value of the model was 0.002 14, less than the critical value p=0.05, indicating that this model was also significant. Between the two variables that had a significant effect on lignin removal (calcium hydroxide loading and temperature), the effect of calcium hydroxide loading was also more significant (p<0.0001, regression coefficient was 4.79). To explore the relationship between xylan removal and lignin removal, Origin 9 was used to make scatter plot and regression analysis for xylan removal and lignin removal, with results shown in Figure 2.

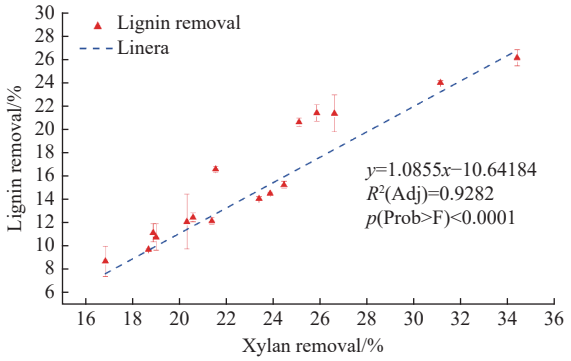


Figure 2 Relationship and regression analysis between xylan removal and lignin removal during lime pretreatment

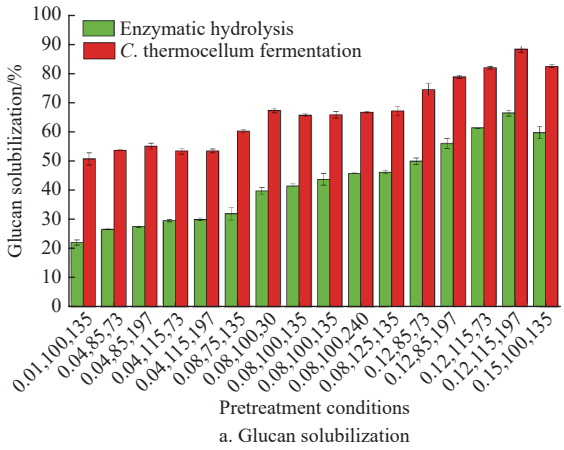
Regression analysis showed that there was a significant linear correlation between xylan removal and lignin removal. The determination coefficient R^2 of the model was 0.9282. The p value of the model was less than 0.0001, indicating that this model was significant. This meant that with the intensification of lime pretreatment conditions, more lignin could be removed to increase the carbohydrate solubilization of subsequent enzymatic hydrolysis and *C. thermocellum* fermentation. However, this would be accompanied by releasing more xylan into the pretreatment hydrolysate, which could lead to more xylooligomer degradation if exposed in alkaline condition for long duration[50].

3.4 Enzymatic hydrolysis and *C. thermocellum* fermentation of lime pretreated maize stover

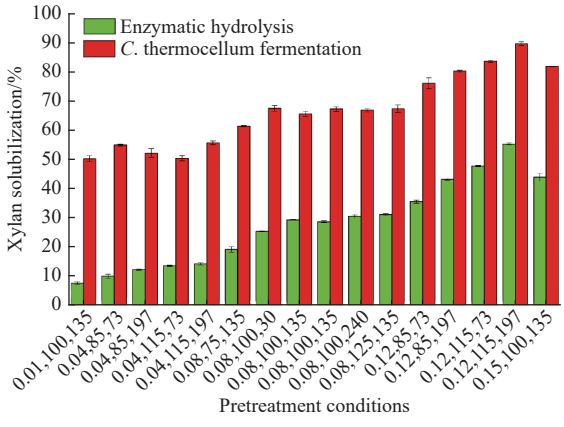
The amenability of pretreated maize stover for conversion by enzymatic hydrolysis featuring fungal cellulase and *C. thermocellum* fermentation was evaluated in terms of carbohydrate (glucan and xylan) solubilization. Figure 3 summarized the carbohydrate solubilization of pretreated maize stover by enzymatic hydrolysis and *C. thermocellum* fermentation (see Table A2 for more data).

For enzymatic hydrolysis, glucan solubilization was 22.18% to 66.21%, while xylan solubilization was 7.53% to 55.04%. At the pretreatment conditions of 0.01 g/g [$Ca(OH)_2$/dry maize stover], 100°C and 135 min, solubilization of glucan and xylan by enzymatic hydrolysis were both at the minimum. With the intensification of pretreatment conditions, carbohydrate solubilization increased gradually. At the pretreatment conditions of 0.12 g/g [$Ca(OH)_2$/dry maize stover], 115°C and 197 min, solubilization of glucan and xylan by enzymatic hydrolysis reached the maximum. Under the same pretreatment conditions, solubilization of glucan and xylan for *C. thermocellum* fermentation was also at the minimum and maximum respectively. For *C.*

thermocellum fermentation, glucan solubilization was 50.58% to 87.94% and xylan solubilization was 50.07% to 89.33%. As listed in Table A2 and Figure 3, solubilization of glucan and xylan for *C. thermocellum* fermentation was higher than that of enzymatic hydrolysis under the same pretreatment conditions. Compared to enzymatic hydrolysis, glucan solubilization for *C. thermocellum* fermentation increased by 1.3 folds and xylan solubilization increased by 5.7 folds when pretreatment conditions were 0.01 g/g [$Ca(OH)_2$/dry maize stover] at 100°C and 135 min. With the intensification of pretreatment conditions, the difference in carbohydrate solubilization between enzymatic hydrolysis and *C. thermocellum* fermentation reduced gradually. When the pretreatment conditions were 0.12 g/g [$Ca(OH)_2$/dry maize stover], 115°C and 197 min, the difference in carbohydrate solubilization reached the lowest value, with glucan solubilization increased by 0.3 folds and xylan solubilization increased by 0.6 folds. The above results indicated that *C. thermocellum* fermentation had excellent carbohydrate solubilization ability compared to enzymatic hydrolysis, particularly for pretreatment under relatively mild conditions[29,30]. Pretreatment had stronger effects in the increase of carbohydrate solubilization for enzymatic hydrolysis. The reason for higher carbohydrate solubilization particularly under less severe pretreatment conditions for *C. thermocellum* fermentation could be that its cellulosome enzyme system was more effective than fungal cellulase system in terms of higher specific enzyme activity and enzyme diversity[51].



a. Glucan solubilization

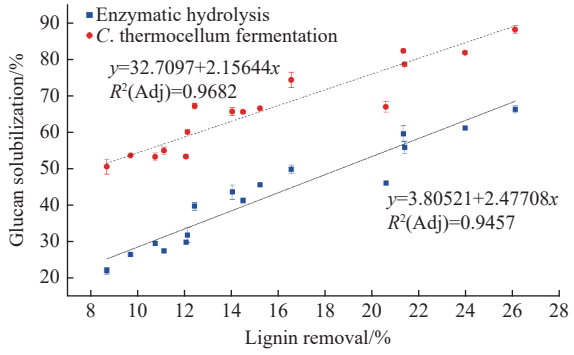


b. Xylan solubilization

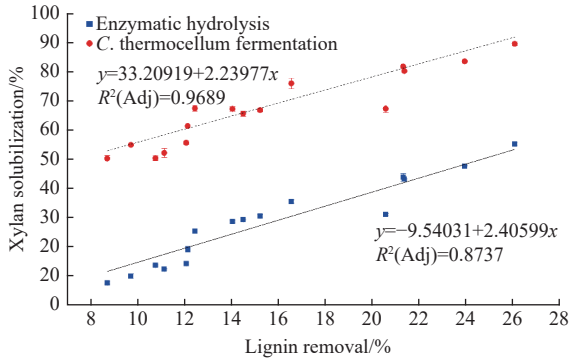
Figure 3 Carbohydrate solubilization by enzymatic hydrolysis and *C. thermocellum* fermentation under various pretreatment conditions

Statistical analysis for carbohydrate solubilization of lime pretreated maize stover by the two solubilization systems was listed in Table A3. Quadratic polynomial regression equations were used to fit the carbohydrate solubilization results. Analysis of variance showed that the determination coefficient R^2 values of the four models were between 0.9497 and 0.9733. The p values of the four models were all less than 0.05, indicating the suitability of the four quadratic polynomial regression models. For carbohydrate solubilization by enzymatic hydrolysis, the effects of $Ca(OH)_2$ loading and temperature were both significant (p<0.05), which was consistent with some previous studies on alkali pretreatment of lignocellulose[52,53,31]. The effect of time was not significant for the confidence interval of 0.05, possibly due to the fact that the selected pretreatment durations were all long enough. For carbohydrate solubilization by *C. thermocellum* fermentation, only $Ca(OH)_2$ loading was found to be significant (p<0.0001). For *C. thermocellum* fermentation, neither the effect of temperature nor the effect of time was significant for the confidence interval of 0.05. One explanation was that *C. thermocellum* fermentation was capable of achieving significant carbohydrate solubilization (>50%) even under the mildest pretreatment conditions, and the selected pretreatment durations were all long enough and temperature were all high enough.

To investigate the relationship between lignin removal by lime pretreatment and carbohydrate solubilization by the two systems, Origin 9 was used to make scatter plot and regression analysis, and the results were shown in Figure 4.



a. Glucan solubilization



b. Xylan solubilization

Figure 4 Relationship and regression analysis between lignin removal and carbohydrate solubilization by enzymatic hydrolysis and *C. thermocellum* fermentation

Regression analysis showed that there was a significant linear correlation (p<0.0001) between lignin removal by lime pretreatment and carbohydrate solubilization by the two systems. As shown in Figure 4a and 4b, the determination coefficient R^2 of the four linear regression models ranged from 0.8737 to 0.9689. For glucan and xylan solubilization, the slopes of the linear regression equations for enzymatic hydrolysis were both higher than those for *C.*

thermocellum fermentation, which again suggested that carbohydrate solubilization by enzymatic hydrolysis was affected by pretreatment (i.e., lignin removal) more than that of *C. thermocellum* fermentation. With respect to the intercepts of the linear regression equations, carbohydrate solubilization by enzyme hydrolysis was much less than that for *C. thermocellum* (28.9% less for glucan solubilization and 42.8% less for xylan solubilization). This explained that compared to commercial fungal cellulase at relatively high enzyme loading (10 mg/g solids), *C. thermocellum fermentation* had better performance for carbohydrate solubilization. Figure 4 and Table A2 showed that close to 90% of carbohydrate solubilization could be reached with 26% lignin removal by lime pretreatment at 0.12 g/g [$Ca(OH)_2$/dry maize stover], 115°C and 197 min.

4 Conclusions

Lime pretreatment resulted in relative increase of glucan, xylan and lignin contents in the solid fraction of pretreated maize stover. Carbohydrate recovery was at least 85% for various pretreatment conditions. Lime loading was the most influencing factor for lignin and xylan removal during pretreatment. Xylan removal and lignin removal was found to have a linear correlation. *C. thermocellum* resulted in much higher carbohydrate solubilization than enzymatic hydrolysis by fungal celluases for solids from all the pretreatment conditions. Carbohydrate solublization by both conversion systems was found to have a linear correlation with lignin removal. Less severe pretreatment was required for *C. thermocellum* with the same carbohydrate solubilization.

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[References]

[1] Wang F Q, Ouyang D H, Zhou Z Y, Page S J, Liu D H, Zhao X B. Lignocellulosic biomass as sustainable feedstock and materials for power generation and energy storage. Journal of Energy Chemistry, 2021; 57: 247–280.

[2] Macrae R, Robison R, Sadler M. Encyclopedia of food science, food technology and nutrition. Agricultural and Food Sciences, Chemistry, Academic Press, 1993.

[3] Lynd L R, Weimer P J, Zyl W H v, Pretorius I S. Microbial cellulose utilization: Fundamentals and biotechnology. Microbiology and Molecular Biology Reviews, 2002; 66(3): 506–577.

[4] Xiao Y, Liu Y J, Feng Y G, Cui Q. Progress in synthetic biology research of Clostridium thermocellum for biomass energy applications. Synthetic Biology Journal, 2023; 4(6): 1055–1081. (in Chinese)

[5] Wyman C. Handbook on bioethanol: Production and utilization. New York: Routledge. 1996; 444 p.

[6] Alvira P, Tomás-Pejó E, Ballesteros M, Negro M J. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour Technol, 2010; 101(13): 4851–4861.

[7] Yu Z, Jameel H, Chang H-M, Park S. The effect of delignification of forest biomass on enzymatic hydrolysis. Bioresour Technol, 2011; 102(19): 9083–9089.

[8] Chang V S, Holtzapple M T. Fundamentals factors affecting biomass reactivity. App.Biochem Biotechnol, 2000; 84: 5–37.

[9] Berlin A, Gilkes N, Kurabi A, Bura R, Tu M B, Kilburn D, et al. Weak lignin-binding enzymes: a novel approach to improve activity of cellulases for hydrolysis of lignocellulosics. App.Biochem Biotechnol, 2005; 121: 163–170.

[10] Hendriks A T W M, Zeeman G. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour Technol, 2009; 100(1): 10–18.

[11] Shao X J, Jin M J, Guseva A, Liu C G, Balan V, Hogsett D, et al. Conversion for Avicel and AFEX pretreated corn stover by *Clostridium thermocellum* and simultaneous saccharification and fermentation: insights into microbial conversion of pretreated cellulosic biomass. Bioresour Technol, 2011; 102(17): 8040–8045.

[12] Hsu T A. Pretreatment of biomass. In: Wyman C (Ed.). Handbook on Biomass Ethanol. Washington DC: Taylor and Francis. 1996; pp.179–212.

[13] Mosier N, Wyman C, Dale B, Elander R, Lee Y Y, Holtzapple M, et al. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol, 2005; 96(6): 673–686.

[14] Wyman C E, Dale B E, Elander R T, Holtzapple M, Ladisch M R, Lee Y Y. Coordinated development of leading biomass pretreatment technologies. Bioresour Technol, 2005; 96(18): 1959–1966.

[15] Li X J, Xu Z X, Yu J M, Huang H, Jin M J. In *situ* pretreatment during distillation improves corn fiber conversion and ethanol yield in the dry mill process. Green Chem, 2019; 21(5): 1080–1090.

[16] Kim J S, Lee Y Y, Kim T H. A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. Bioresour Technol, 2015; 199: 42–48.

[17] Park Y C, Kim J S. Comparison of various alkaline pretreatment methods of lignocellulosic biomass. Energy, 2012; 47(1): 31–35.

[18] Kim T H. Pretreatment of lignocellulosic biomass. In: Yang S-T, El-Enshasy H A, Thongchul N (Eds.). Bioprocessing Technologies in Biorefinery for Sustainable Production of Fuels, Chemicals, and Polymers. New York: John Wiley&Sons. 2013; pp.91–109. doi: 10.1002/9781118642047.ch6.

[19] Bhardwaj N, Kumar B, Agrawal K, Verma P. Current perspective on production and applications of microbial cellulases: a review. Bioresources and Bioprocessing, 2021; 8: 95.

[20] Murashima K, Nishimura T, Nakamura Y, Koga J, Moriya T, et al. Purification and characterization of new endo-1, 4-β-d-glucanases from *Rhizopus oryzae*. Enzyme Microb Tech, 2002; 30(3): 319–326.

[21] Dashtban M, Maki M, Leung K T, Mao C Q, Qin W S. Cellulase activities in biomass conversion: measurement methods and comparison. Crit Rev Biotechnol, 2010; 30(4): 302–309.

[22] Yuan Y, Zhai R, Li Y, Chen X X, Jin M J. Developing fast enzyme recycling strategy through elucidating enzyme adsorption kinetics on alkali and acid pretreated corn stover. Biotechnology for Biofuels, 2018; 11: 316.

[23] Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons B A, Blanch H W. The challenge of enzyme cost in the production of lignocellulosic biofuels. Biotechnol Bioeng, 2012; 109(4): 1083–1087.

[24] Lynd L R, Liang X Y, Biddy M J, Allee A, Cai H, Foust T, et al. Cellulosic ethanol: Status and innovation. Curr Opin Biotechnol, 2017; 45: 201–211.

[25] Maki M, Leung K T, Qin W S. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int J Biol Sci, 2009; 5(5): 500–516.

[26] Zhao X-H, Wang W, Tong B, Zhang S-P, Wei D-Z. A newly isolated penicillium oxalicum 16 cellulase with high efficient synergism and high tolerance of monosaccharide. App.Biochem Biotechnol, 2015; 178(1): 173–183.

[27] Hirano K, Kurosaki M, Nihei S, Hasegawa H, Shinoda S, Haruki M, et al. Enzymatic diversity of the *Clostridium thermocellum* cellulosome is crucial for the degradation of crystalline cellulose and plant biomass. Sci Rep, 2016; 6: 35709.

[28] Xu Q, Resch M G, Podkaminer K, Yang S H, Baker J O, Nonohoe B S, et al. Dramatic performance of *Clostridium thermocellum* explained by its wide range of cellulase modalities. Sci Adv, 2016; 2(2): e1501254.

[29] Paye J M D, Guseva A, Hammer S K, Gjersing E, Davis M F, Davison B H, et al. Biological lignocellulose solubilization: comparative evaluation of biocatalysts and enhancement via cotreatment. Biotechnology for Biofuels, 2016; 9: 8.

[30] Papathoti N K, Mendam K, Thepbandit W, Burgula N, Sangpueak R, Saengchan C, et al. Bioethanol production from alkali-pretreated cassava stem waste via consolidated bioprocessing by ethanol-tolerant *Clostridium thermocellum* ATCC 31, 924. Biomass Conv. Bioref, 2022; 14: 6821–6833.

[31] Chen Y, Stevens M A, Zhu Y M, Holmes J, Xu H. Understanding of alkaline pretreatment parameters for corn stover enzymatic saccharification. Biotechnology for Biofuels, 2013; 6: 8.

[32] Liang Y G, Zheng Z, Luo X Z, Si Y B, Cao D J, Nie E, et al. Lime pretreatment to improve methane production of smooth cordgrass (*spartina*

alterniflora). Chem Eng J, 2013; 217: 337–344.
[33] Choi W I, Park J Y, Lee J P, Oh Y K, Park Y C, Kim J S, et al. Optimization of NaOH-catalyzed steam pretreatment of empty fruit bunch. Biotechnology for Biofuels, 2013; 6: 170.
[34] Yoo C G, Nghiem N P, Hicks K B, Kim T H. Pretreatment of corn stover using low-moisture anhydrous ammonia (LMAA) process. Bioresour Technol, 2011; 102: 10028–10034.
[35] Chen Y, Sharma-Shivapp.R R, Keshwani D, Chen C C. Potential of agricultural residues and hay for bioethanol production. App.Biochem Biotechnol, 2007; 142: 276–290.
[36] Yang B Y, Montgomery R. Alkaline degradation of glucose: Effect of initial concentration of reactants. Carbohyd Res, 1996; 280(1): 27–45.
[37] Knill C J, Kennedy J F. Degradation of cellulose under alkaline conditions. Carbohyd Polym, 2003; 51(3): 281–300.
[38] Stoklosa R J, Hodge D B. Fractionation and improved enzymatic deconstruction of hardwoods with alkaline delignification. BioEnerg Res, 2015; 8: 1224–1234.
[39] Yu H B, Du W Q, Zhang J, Ma F Y, Zhang X Y, Zhong W X. Fungal treatment of cornstalks enhances the delignification and xylan loss during mild alkaline pretreatment and enzymatic digestibility of glucan. Bioresour Technol, 2010; 101(17): 6728–6734.
[40] Chen X X, Zhai R, Shi K Q, Yuan Y, Dale B E, Gao Z, et al. Mixing alkali pretreated and acid pretreated biomass for cellulosic ethanol production featuring reduced chemical use and decreased inhibitory effect. Ind Crop Prod, 2018; 124: 719–725.
[41] Pedersen M, Viksø-Nielsen A, Meyer A S. Monosaccharide yields and lignin removal from wheat straw in response to catalyst type and pH during mild thermal pretreatment. Process Biochem, 2010; 45(7): 1181–1186.
[42] Poddar B J, Nakhate S P, Gupta R K, Chavan A R, Singh A K, Khardenavis A A, et al. A comprehensive review on the pretreatment of lignocellulosic wastes for improved biogas production by anaerobic digestion. Int J Environ Sci Technol, 2022; 19: 3429–3456.
[43] Petersson G. Branched deoxyaldaric acids from alkaline degradation of carbohydrates: structure determination by mass spectrometry of trimethylsilyl derivatives. Carbohyd Res, 1975; 43(1): 1–8.
[44] Li J, Miao G H, He L, Chen K L, Gaun Q Q, Qian W M, et al. Analyzing the delignification, carbohydrate degradation kinetics, and mechanism of wet-storage bagasse in oxygen-alkali cooking. Cellulose, 2022; 29: 9421–9435.
[45] Alvarez-Vasco C, Zhang X. Alkaline hydrogen peroxide pretreatment of softwood: hemicellulose degradation pathways. Bioresour Technol, 2013; 150: 321–327.
[46] Shen J C, Wyman C E. A novel mechanism and kinetic model to explain enhanced xylose yield from dilute sulfuric acid compared to hydrothermal pretreatment of corn stover. Bioresour Technol, 2011; 102(19): 9111–9120.
[47] Xu J L, Cheng J J, Sharma-Shivapp.R R, Burns J C. Lime pretreatment of switchgrass at mild temperatures for ethanol production. Bioresour Technol, 2010; 101(8): 2900–2903.
[48] Toledano A, Serrano L, Labidi J. Organosolv lignin depolymerization with different base catalysts. J Chem Technol Biot, 2012; 87(11): 1593–1599.
[49] Kirui A, Zhao W C, Deligey F Yang H, Kang X, Mentink-Vigier F, et al. Carbohydrate-aromatic interface and molecular architecture of lignocellulose. Nature Communications, 2022; 13: 538.
[50] Gupta R, Lee Y Y. Investigation of biomass degradation mechanism in pretreatment of switchgrass by aqueous ammonia and sodium hydroxide. Bioresour Technol, 2010; 101(21): 8185–8191.
[51] Feng Y G, Liu Y J, Cui Q. Research progress in cellulosomes and their applications in synthetic biology. Synthetic Biology Journal, 2022; 3(1): 138–154. (in Chinese)
[52] Zhong L, Wang C, Xu M M, Ji X X, Yang G H, Chen J H, et al. Alkali-catalyzed organosolv pretreatment of lignocellulose enhances enzymatic hydrolysis and results in highly antioxidative lignin. Energy & Fuels, 2021; 35: 5039–5048.
[53] Mao J H, Xiao C, Gong D H, Zhu J W, Qian J. Study on mechanism of cellulose hydrolysis during alkali thermal pretreatment of lignocellulose by density functional theory. Biomass and Bioenergy, 2023; 172: 106754.

Appendix

Table A1 Statistical Analysis for Removal of Xylan and Lignin[a]

	Xylan removal		Lignin removal	
R-Squared	0.930		0.955	
Prob>F	0.0075		0.002 14	
Terms	Estimate[b]	*p*-value	Estimate[b]	*p*-value
A-lime loading	3.93	0.0003	4.790	<0.0001
B-temperature	2.01	0.01	2.070	0.0054
C-time	1.05	0.1001	1.050	0.0746
AB	1.89	0.037	1.260	0.0946
AC	0.92	0.2414	0.530	0.4406
BC	0.17	0.8200	–0.350	0.6035
A^2	–0.41	0.5566	0.570	0.3700
B^2	0.13	0.8532	1.050	0.1269
C^2	–0.13	0.8520	0.160	0.8003

Note: [a] Statistical analysis was performed in Design-Expert v10. [b] Coefficients of quadratic polynomial regression equations.

Table A2 Carbohydrate Solubilization of Pretreated Solids by Enzymatic Hydrolysis and *C. thermocellum Fermentation*

Std Sequence	Lime loading [$Ca(OH)_2$/dry maize stover]/(g·g^{-1})	Pretreatment Temp/°C	Pretreatment Time/min	Enzymatic hydrolysis Solubilization/%		*C. thermocellum* fermentation Solubilization/%	
				Glucan	Xylan	Glucan	Xylan
9	0.01	100	135	22.18	7.53	50.58	50.07
1	0.04	85	73	26.58	9.92	53.60	54.71
5	0.04	85	197	27.57	12.20	54.92	51.97
3	0.04	115	73	29.58	13.49	53.24	50.18
7	0.04	115	197	29.95	14.11	53.34	55.44
11	0.08	75	135	31.90	19.06	60.02	61.18
13	0.08	100	30	39.76	25.21	67.07	67.24
15	0.08	100	135	41.35	29.15	65.51	65.40
16	0.08	100	135	43.66	28.50	65.61	67.06
14	0.08	100	240	45.65	30.39	66.47	66.63
12	0.08	125	135	46.09	31.00	66.86	67.10
2	0.12	85	73	49.84	35.36	74.21	75.78
6	0.12	85	197	55.84	42.99	78.47	79.99
4	0.12	115	73	61.14	47.52	81.67	83.33
8	0.12	115	197	66.21	55.04	87.94	89.33
10	0.15	100	135	59.55	43.70	82.16	81.56

Table A3 Statistical Analysis of Carbohydrate Solubilization of Pretreated Solids by Enzymatic Hydrolysis and *C. thermocellum Fermentation*[a]

	Enzymatic hydrolysis				*C. thermocellum* fermentation			
	Glucan solubilization		Xylan solubilization		Glucan solubilization		Xylan solubilization	
R-Squared	0.9733		0.9574		0.9652		0.9497	
Prob>F	0.0005		0.0018		0.001		0.003	
Terms	Estimate	*p*-value	Estimate	*p*-value	Estimate	*p*-value	Estimate	*p*-value
A-lime loading	13.34	<0.0001	14.06	<0.0001	11.740	<0.0001	12.38	<0.0001
B-temperature	3.73	0.0079	3.64	0.0291	1.940	0.0848	1.89	0.1660
C-time	1.64	0.1368	1.96	0.1760	0.800	0.4270	0.86	0.5014
AB	2.04	0.1533	2.34	0.2104	2.360	0.1035	2.24	0.2017
AC	1.21	0.3676	1.53	0.3938	1.140	0.3898	0.96	0.5618
BC	–0.19	0.882	–0.22	0.8987	0.098	0.9392	1.22	0.4642
A^2	0.022	0.9855	–0.47	0.7700	0.670	0.5788	0.32	0.8338
B^2	–0.64	0.6009	–0.68	0.6764	–0.360	0.7603	–0.27	0.8569
C^2	0.67	0.5825	0.30	0.8534	0.810	0.5043	0.72	0.6402

Note: a Statistical analysis was performed in Design-Expert v10.