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Two-Stage Acidic–Alkaline Hydrothermal Pretreatment of Lignocellulose for the High Recovery of Cellulose and Hemicellulose Sugars

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Abstract The focus of this work was to develop a combined acid and alkaline hydrothermal pretreatment of lignocellulose that ensures high recovery of both hexose and pentose. Dilute sulfuric acid and lime pretreatments were employed sequentially. Process performance was optimized in terms of catalyst concentration, retention time, and temperature using response surface methodology. Medium operational conditions in the acid stage and harsh conditions in the alkaline stage were desirable with optimal performance at 0.73 wt% H_2SO_4, 150 °C, 6.1 min in the first stage, and 0.024 g lime/g biomass, 202 °C, 30 min in the second stage. In comparison to single-stage pretreatments with high recovery of either glucose or xylose, two-stage process showed great promises with >80 % glucose and >70 % xylose recovery. In addition, the method greatly improved ethanol fermentation with yields up to 0.145 g/g *Miscanthus*, due to significantly reduced formation of inhibitory by-products such as weak acids, furans, and phenols. Supplementing biomimetic acids would further increase glucose yield by up to 15 % and xylose yield by 25 %.

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Introduction

Utilizing lignocellulosic biomass as sustainable material has lately become a compelling alternative among conversion technologies in the biofuels and bio-based industry. Widely distributed and largely untapped, lignocellulose can continuously provide low-cost feedstock [1], which would avoid disturbing the food supply as is the problem with conventional biofuels. On the other hand, lignocellulose derived biofuels are not yet commercially feasible, due to the associated prohibitive conversion and feedstock logistics costs [2]. Recently, it has been noticed that the unfavorable process economics can be improved by means of efficient co-utilization of cellulose and hemicellulose instead of cellulose fraction alone which was focused in the past [3]. However, the stringent requirement of utilizing all lignocellulose components would impose great challenges on the existing conversion processes, especially the initial pretreatment step. Previously, the pretreatment process was designed with the major objective of effective cellulose recovery, and accordingly, a variety of pretreatment methods have been developed including physical, chemical, physicochemical, and biological methods and their combinations [4]. Unfortunately, meanwhile, none of these methods can obtain high sugar recovery extensively from hemicellulose [5].

To achieve maximum multiple sugar yields simultaneously, pretreatment streamline was suggested to be divided into separate stages [6, 7]. It was well known that the severity of pretreatment conditions greatly affects the hydrolysis of lignocellulose components, especially hemicellulose [8]. A severe condition would cause significant degradation of hemicellulose sugars into inhibitory compounds, while a relatively high degree of severity is still desirable to enhance the enzymatic digestibility of cellulose. Therefore, in the separate pretreatment process, varied severities were applied, where the first stage was conducted at low severity for efficient hemicellulose hydrolysis, and another stage under more severe conditions was followed to treat the remaining residue [9]. In addition to different severities application, distinctive pretreatment methods were conducted in each stage to further improve the overall biomass utilization. This fractionation strategy was based on an essential feature that most pretreatment methods have varied preference to treat certain specific components. As such, acid pretreatment can be used to mainly hydrolyze hemicellulose while alkaline pretreatment to efficiently modify or remove lignin [3]. Up to date, the scheme of sequential acid and alkaline pretreatment was investigated the most. A wide range of promising pretreatment methods have been employed including dilute acid hydrolysis, steam explosion, and hot water treatment in the acid stage succeeded by ammonia, alkaline peroxide treatment, and Organoslov process in the alkaline stage [10–13]. Many of them proved significantly improved yields of both cellulose and hemicellulose sugars and required fewer enzymes for hydrolysis than single-stage pretreatments.

Although the previous studies on two-stage pretreatments have verified the above-shown benefits, the effects of pretreatment conditions on the production of important hydrolysis products and the overall performance were still not well known. Additionally, there was lack of the basic knowledge of the degradation profiles and fates for major lignocellulose components throughout two-stage processes. All these absent information would be necessary for in-depth understanding of pretreatment mechanism and further process improvement of two-stage methods.

To bridge the knowledge gap, in this study, ACidic–ALkaline pretreatments in succession (ACAL pretreatment) were developed. The two-stage process was carried out with acid pretreatment at low severity in the first stage mainly for hemicellulose hydrolysis and then obtained efficient lignin removal and greatly enhanced cellulose digestibility in the second stage via alkaline pretreatment at elevated severity level. To make the process more commercially feasible, commonly applied dilute acid and lime pretreatments were utilized in each stage, respectively. The process was optimized by using response surface methodology (RSM) analysis. Finally, under the optimal conditions, two-stage acidic–alkaline pretreatments were compared with single-stage acid and alkaline pretreatments in terms of pretreatment effectiveness. The objective of this study was to evaluate the influence of major pretreatment conditions on ACAL process, quantitatively characterize the biomass components degradations, and clearly identify the advantages of ACAL process over single-stage pretreatments.

Materials and Methods

Raw Material

Miscanthus was used in this research as the model feedstock. The material was harvested in spring 2008 on the farm in Urbana, IL, and then air-dried below 45 °C to obtain dry matter content between 91 % and 94 %. The dried material was hammermilled, and the fraction passing through ¼-in. (6.35 mm) sieve was collected and analyzed for its contents of major components according to the National Renewable Energy Laboratory (NREL) standard procedures (Technical Report NREL/TP-510-42618). The chemical composition of the dry-based *Miscanthus* was 39.2±0.3 % glucan, 19.5±0.4 % xylan, 1.2±0.1 % arabinan, and 24.2±1.1 % lignin.

Pretreatment Setup and Operation

In the first stage of acid pretreatment, experiments were carried out in a batch reactor (Model 4534, PARR Instrument Co., Moline, IL) equipped with 2 L cylindrical pressure vessel (9.5 cm i.d.). One hundred twenty grams of dry-based *Miscanthus* samples were loaded for each batch with various acid solutions to keep a fixed solid loading of 20 % by weight. The pretreatment applied pure sulfuric acid solutions and sulfuric acid solutions mixed with biomimetic acids individually. The biomimetic acids used in this study were trifluoroacetic acid (TFA) and maleic acid (MA). Preceding the reaction in the vessel, the biomass was steeped in the acid solutions for 9 h at ambient temperature. After loaded with the reactants, the vessel was clamped shut and then heated at 6–8 °C/min. Counting of the reactions was started once the vessel reached the desired temperature, and the vessel was controlled at a constant temperature and pressure with agitation at 400 rpm. Once the pretreatment finished, the system was cooled down to 60 °C in about 10 min, and the pressure was released immediately thereafter. After completion of the acid pretreatment, the solids and liquids were separated through Whatman No.1 filter paper. Hydrolysates (liquid fractions) were stored for chemical analysis and further use in the fermentation tests. Solid residues were air-dried at 37 °C till reaching 90–95 % dry matter contents and then used in the second-stage alkaline pretreatment.

A different batch reactor (Model 4593, PARR Instrument Co., Moline, IL) was set up for the second-stage pretreatment with 100 mL cylinder-shaped pressure vessel (3.3 cm i.d.).

The operation procedure of the reactor was the same as that of the acid pretreatment reactor. Differently, 6 g of dried solid residues from first-stage pretreatment were loaded with lime solution to bring the solid loading to 20 % by weight. After the second-stage reaction, the reacted biomass was filtered, and the liquid fractions were collected for chemical analysis. Solid residues were tested for enzymatic digestibility and blended with first-stage hydrolysates accordingly for fermentation tests.

Experimental Design and Statistical Analysis

The central composite design, which is the standard RSM, was applied in both stages separately for optimization of the pretreatment conditions. In the acid stage, acid dosage, temperature, and residence time were taken as the independent variables, since it has been found that the process chemistry during hemicellulose hydrolysis greatly depended on these three factors [14]. On the contrary, it has been observed that, during the lime pretreatment at temperature higher than 80 °C, residence time had little effect on glucose yield if longer than 30 min [15, 16]. Therefore, in the optimization study of the second stage, lime loading and temperature were selected as two independent variables, with a fixed residence time of 30 min. Each independent variable at both stages was investigated at five levels. The variables were coded at the beginning to exclude the effect of their individual values under different units. The ranges and levels of the variables were given in Table 1. Yields of sugars (glucose and xylose), furans [furfural and hydroxymethylfurfural (HMF)], weak acids (acetic, formic, and levulinic acids), and total phenols were selected as the response variables, respectively. The response variables were approximated by a second-order Taylor expansion:

$$y = \beta_0 + \sum \beta_i x_i + \sum \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 + e \quad (1)$$

Where y is the predicted response, x_i and x_j are coded values of the independent variables, β_0, β_i, β_{ij}, and β_{ii} are the Taylor expansion coefficients, and e is the error of the fitted model.

The regression and statistical analysis were carried out using Microsoft Origin 8.0, and the visualization of response surfaces were displayed by MATLAB 7.13.

For furans and weak acids which contain multiple responses, a composite response surface was derived to locate the best compromise among the responses through desirability function approach [17]. In this approach, all related responses were weighed together into one criterion, an overall desirability function, which was then optimized by RSM. The

Table 1 Coded values of the tested variables at various levels

Acid stage	Variables	Range and levels				
		−2	−1	0	1	2
	Acid dosage (wt% H_2SO_4)	0.25	0.50	0.75	1.00	1.25
	Temperature (°C)	130	145	160	175	190
	Residence time (min)	5	15	25	35	45
Alkaline stage	Variables	Range and levels				
		−1.414	−1	0	1	1.414
	Lime loading (g $Ca(OH)_2$/g biomass)	0	0.0117	0.04	0.0683	0.08
	Temperature (°C)	175	185	210	235	245

overall desirability (D) was calculated as a geometric mean of all individual desirabilities (d_i) by different weight depending on its importance to the response as follows:

$$D = \left(d_1^{w1} \times d_2^{w2} \times \ldots \times d_m^{wm}\right)^{1/(w1+w2+\ldots+wm)} \quad (2)$$

Where wi ($1 \le i \le m$) is the weight factor for each desirability. In the study, we assumed all related individual by-products (furans and weak acids) contributed equally to the overall adverse effect on fermentation, and their own inhibitory effect was employed to interpret the individual desirability [18].

In this work, most pretreatment tests and all fermentation experiments were carried out in duplicate, while enzymatic hydrolysis was performed in triplicate. A 95 % confidence level was applied for data analysis.

Enzymatic Hydrolysis

The pretreated solid materials were enzymatically hydrolyzed following the NREL standard procedure (Technical Report NREL/TP-510-42629). Hydrolysis was conducted in 50 mM sodium citrate buffer (pH 4.8) at the loading of 1.0 wt% glucan content. Applied enzyme loadings were 15 FPU/g glucan of cellulase (Spezyme CP, Genencor), 2 CBU/FPU of β-glucosidase (Novozym 188, Sigma-Aldrich) supplemented with xylase (Multifect Xylanase, Genencor). The test flasks were incubated at 50 °C for 72 h, and hydrolysates were sampled every 24 h.

Simultaneous Saccharification and Co-Fermentation (SSCF)

Saccharomyces cerevisiae DA2416 was used as the host strain for producing ethanol from xylose and glucose in the pretreated hydrolysates. Methods for strain cultivation were described previously [19]. Simultaneous saccharification and co-fermentation (SSCF) was carried out in 250 mL flasks containing 50 mL of YP (1 % *w/v* yeast extract, 2 %*w/v* peptone) with pretreated *Miscanthus* slurry including solid residue and hydrolysate (10 % *w/v* solid loading) at 30 °C and 100 rpm. The initial pH of medium was adjusted to 5.0±0.1 through overliming (addition of $Ca(OH)_2$ to pH 10–11 first, followed by H_2SO_4 down to pH 5). Yeast was inoculated with an initial cell concentration of 0.35 g/L. During SSCF, Spezyme cellulose cocktail (30 FPU/g hydrolysate), Novozyme 188 β-glucosidase (60 CBU/g hydrolysate) and Multifect xylanase (0.25 mL/g hydrolysate) were supplemented for saccharification of hydrolysate. After 48 h of SSCF, newly cultured cells (0.35 g/L) were added in order to enhance sugars consumption.

Analytical Methods

For pretreatment and enzymatic hydrolysis tests, the concentrations of monosaccharides, furans, and weak acids were measured using a high-performance liquid chromatography (HPLC) system (Shimadzu) equipped with a refractive index detector (Waters) as described previously [20]. Oligosaccharides in the hydrolysates were broken down to monosaccharides through 4 % *w/w* sulfuric acid hydrolysis at 121 °C for 60 min for quantitative analysis by HPLC. Hydrolysates after pretreatment were analyzed for phenolic compounds by gas chromatography-mass spectrometry (GC-MS) system according to previously reported methods [20]. Prior to the analysis, hydrolysate samples were extracted with ether twice at 3:1, and subsequently, the ether phase was concentrated by nitrogen bubbling. In addition,

total phenols of the hydrolysates were determined using the Folin–Ciocalteu assay [21]. Samples were diluted by water to adjust absorbance in 0.1–0.5, and total phenols were expressed in gallic acid equivalent.

For fermentation tests, glucose, xylose, xylitol, glycerol, acetate, and ethanol concentrations were determined by HPLC system (Agilent Technologies 1200 Series) equipped with a refractive index detector using a REzex ROA-Organic Acid H^+ (8 %) column (Phenomenex Inc., Torrance, CA). The column was eluted with 5 mM sulfuric acid at 0.6 mL/min at 50 °C.

All the chemicals used in the study were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (St. Louis, MO).

In the acid pretreatment, the combined severity factor (CSF) was used to describe the severity level of the pretreatment conditions taking account of the effects of reaction time, temperature, and acid dosage [10–13]. The CSF was defined as:

$$\mathrm{CSF} = \log\{t \exp[(T - T_{\mathrm{ref}})/14.7]\} - \mathrm{pH} \tag{3}$$

where t is hydrolysis time in minutes, TH is temperature in degrees Centigrade, T_{ref} is the reference temperature, $(T_{\mathrm{ref}} = 100^{\circ}C)$, and pH is the acidity of the prehydrolysates.

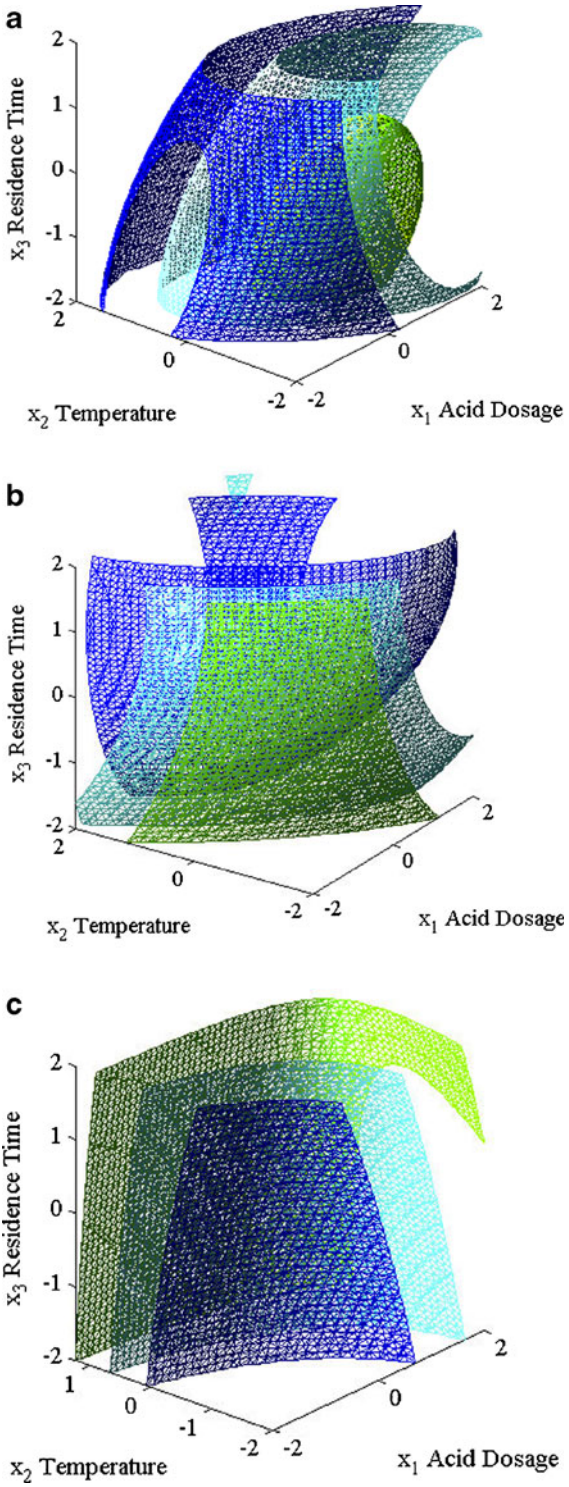
Results and Discussion

The Effect of Pretreatment Conditions on Acid Stage Performance

The contour plots of xylose, furans and acetate, and total phenols in relation to the coded values of three independent variables (acid dosage, temperature, and residence time) were visualized in Fig. 1 and constructed on the basis of fitted quadratic models. The shapes of displayed three-dimensional isocontour surfaces can be understood by combining the commonly plotted two-dimensional response surfaces with the canonical analysis results, and the isocontours described the straightforward interactions among three variables [17].

As can be observed in Fig. 1a, the isocontours of solubilized xylose yield described a score of partial concentric elliptic shells. Under mild conditions, xylose yield increased with all three variable values, but further raising the levels of operational variables into harsher conditions would result in evident drop in xylose yield. General ranges of acid dosage in 0.8–1.0 wt% sulfuric acid, temperature 145–155 °C, and residence time less than 30 min were desirable to obtain the maximized xylose recovery during the pretreatment. The optimal conditions can be achieved at the center of ellipsoids [coded values of (0.58, −0.58, −0.97)] at 0.90 wt%, 151 °C, and 15.3 min with maximal xylose recovery of 13.9 % dry biomass (62.5 % theoretical). These conditions were comparable to the optimal ranges of 0.9–1.8 wt%, 140–153 °C, and 6–40 min by dilute acid pretreatment on various biomass in other reports [14, 22–24], although xylose yield was lower than those reported 76–93 % theoretically, probably in favor of their lower applied solid loading (5–7 %). In addition, the optimal combined severity factor (1.7) was also lower than 2.0–2.3, the only reported value by dilute acid pretreatment on *Miscanthus* [25]. Besides, Fig. 1a also

Fig. 1 Contour plots of response surfaces in first-stage acid pretreatment as a function of acid dosage (x_1), ▶ temperature (x_2), and residence time (x_3). **a** Xylose yield in percent dry biomass. Isovalues of the isoresponse contour surfaces—7.0 % in *blue*, 10.0 % in *cyan*, 13.0 % in *green*. **b** Overall desirability of furans and acetate. Isovalues—0.1 in *blue*, 0.3 in *cyan*, 0.5 in *green*. **c** Total phenols in grams per liter in gallic acid. Isovalues—2.5 in *blue*, 3.0 in *cyan*, 3.5 in *green*


a
x3 Residence Time
x2 Temperature
x1 Acid Dosage
b
x3 Residence Time
x2 Temperature
x1 Acid Dosage
c
x3 Residence Time
x2 Temperature
x1 Acid Dosage

presented that the ellipsoids were elongated along the axis of residence time, which indicated less influence of time on xylose yield than the other two parameters. Here, glucose yield was not taken into account for the process optimization in the acid stage, since the primary target was hemicellulose hydrolysis to xylose.

Figure 1b showed contour plots of composite response surface through desirability function approach integrating acetic acid, furfural, and HMF yields. All three hydrolysis by-products would exert evident inhibitory effects on the downstream fermentation. However, at the induced concentration in this study (3.8–15.2 g/L acetic acid, 0.9–13.2 g/L furfural, 0–3.0 g/L HMF), acetic acid presented the greatest inhibition. Additionally, the formation of three by-products increased with pretreatment severity, although as for acetic acid it tended to level off at higher severity level (data not shown). Based on the different inhibitory effects of furans and acetic acid, the impact of furans changed remarkably at greater presence, while that of acetate moved faster at low concentration. When taking account of concentration and individual effect, the composite contour plots described steadily decreasing overall desirability as severity level increased, which meant continuously intensifying inhibitory effects. At low severity, acetic acid contributed the most to the overall desirability change whereas furans took over at high severity. In addition, the isoresponse contour surfaces tuned parallel to the axis of residence time while above 25 min. This implied that any extended reaction time would not significantly affect the hydrolysis after 25 min pretreatment.

Apart from furans and weak acids, a wide range of phenolic compounds formed from lignin breakdown and carbohydrate degradation during acid hydrolysis, most of which were considered potential fermentation inhibitors as well. Total phenols under various conditions were illustrated graphically in Fig. 1c, and the isocontour defined a group of curved surfaces along the axis of residence time. It can be observed that more phenols were generated with increase of operating severity, which suggested harsh pretreatment conditions were inductive to phenols formation. Besides, similarly as in cases of xylose, furans, and acetate, the effect of reaction time on total phenols appeared to be trivial as can be concluded from the observed parallel surfaces along the direction of residence time. Individual phenols were also analyzed for further understanding of phenols production. Table 2 listed major individual phenols with concentrations greater than 10 mg/L in the hydrolysates. Among the eight primary phenolic compounds, p-coumaric acid, ferulic acid, and vanillin constituted the largest fractions. P-coumaric and ferulic acids are the primary block linkage components in herbaceous plants like *Miscanthus*, and the rest are three phenolic aldehydes along with their corresponding carboxylic acids. In fact, these three aldehydes came from the three basic monolignol units in biomass individually, with p-hydroxybenzaldehyde from p-hydroxyphenyl (H), vanillin from guaiacyl (G), and syringaldehyde from syringyl (S) moiety [26], and the phenols profile was consistent to the biomass composition. It was also important to note that the influence of operational conditions on individual phenols varied. For most phenols, harsh conditions would induce their production, and this was in line with the trend of total phenols. By contrast, concentrations of syringaldehyde, p-coumaric, and ferulic acids decreased with increase of severity levels. It was possibly due to the fact that these phenols were further oxidized to carboxylic acids and subsequently broken into smaller phenolic units. They were more reactive and served as reaction intermediates since the attached hydroxyl and methoxy group to the aromatic ring could activate the aromatic ring by electron donation [27].

Up to date, the influence of operational conditions on the performance of dilute acid pretreatment has been intensively studied, but most of them only focused on sugar recovery [14, 24, 28, 29]. Several studies reported on furans and acetate productions, with limited

Table 2 Yields of primary phenols (concentration>5 mg/L) in the hydrolysates (in milligrams per liter)

Conditions	Acid stage			Alkaline stage				
	Medium (0,0,0)	Harsh (1,1,1)	Mild (−1, −1, −1)	Medium (0,0)	Higher lime loading (1.4,0)	Lower lime loading (−1.4,0)	Higher temp. (0,1.4)	Lower temp. (0, −1.4)
P-hydroxybenzaldehyde	5.4	8.6	6.4	4.9	3.1	19.1	1.2	15.4
P-hydroxybenzoic acid	6.3	15.0	4.3					
Vanillin	30.9	97.7	20.2	10.8	4.7	41.7	2.7	17.6
Vanillic acid	16.6	63.0	8.6	1.4	1.6	14.4		7.0
Syringaldehyde	10.3	5.7	10.8	4.0		26.2		7.6
Syringic acid	8.2	27.5	4.0	1.1	1.2	9.0		4.1
P-Coumaric acid	80.9	17.9	131.9	3.4	4.6	9.4		20.6
Ferulic acid	44.8	5.8	33.6					
Syringol				16.1	22.4	9.9	30.2	4.2
Methylhydroquinone				1.4		0.6	17.9	0.2
2-(4-Hydroxyphenyl) propionic acid				4.9	9.2	1.9	2.1	2.8
3-(4-Hydroxyphenyl) propionic acid				1.7	2.3	1.0	9.2	0.2
3-Vanillyl propanol				2.6	2.6	7.0	3.7	1.6
3-Hydroxybenzoic acid				2.1	2.6	4.2	5.7	3.1

Concentrations of syringol down to 3-hydroxybenzoic acid were shown in ratios to 1.67 mg/L phthalic acid. The 1.67 mg/L phthalic acid equals 1.5-4.0 mg/L phenol depending on the phenol type

information provided on the effect by single pretreatment parameter [30, 31], while there is no report on phenols yield. Here, the effect of pretreatment conditions on xylose, furans, acetate, and phenols were described, and their interactive tendencies can be observed when all three graphs in Fig. 1 were put together. It clearly indicated the conditions for maximal xylose yield were not the best pretreatment conditions overall due to strong induction of most inhibitory compounds. In fact, the operational severity leveraged the reaction favorability between hemicellulose decomposition and xylose degradation. Employment of concentrated acid and elevated temperature may provide an acidic environment that accelerates formation of furfural from xylose and induces pyrolysis of lignin into phenolic compounds [22]. In this regard, medium severities would be suggested to obtain acceptably high xylose yield as well as reduced by-products formation that facilitates the xylose fermentation as a whole. In this study, the best pretreatment conditions were located at 0.73 wt%, 150 °C, and 6.1 min. Under these conditions, the pretreatment assured 12.5 % of xylose yield (56.3 % theoretical) and achieved by-products formation of 1.95 g/L furfural, 6.02 g/L acetic acid, and negligible HMF. Furthermore, residence time was found to have little effect on all major products production, so it could be consider least in the further process development of acid pretreatment.

All the quadratic models were tested for adequacy by the analysis of variance. They were highly significant, and the coefficients of determination (R^2) were all above 0.9. The chosen optimal conditions were confirmed by pretreatment tests with variances of all major product yields less than 5 % compared with the model predicted values.

The Effect of Pretreatment Conditions on Alkaline Stage Performance

Under the recommended condition for acid pretreatment, the effectiveness of post-lime pretreatment was evaluated through a 2^2 central composite design, and the response surfaces of major products were illustrated in Fig. 2.

As shown in Fig. 2a, glucose release after enzymatic hydrolysis was mainly affected by temperature but lime loading. Along with temperature increase, glucose yield first increased but then declined. On the other hand, at higher temperature, glucose release was facilitated as lime loading increased, while the opposite tendency was observed at lower temperature. High glucose yield of 0.4 g/g residue can be attained at nearly all applied lime loadings if medium temperature range of 185–220 °C was applied. Contrarily, the profile of weak acids in Fig. 2b was simple. The overall desirability reduced continuously with both lime loading and temperature, which meant generally more acetic, formic, and levulinic acids were induced from the release of acetyl group during hemicellulose and furans degradation. Through the hydrolysis, great presences of acetic and formic acids were detected, with concentrations of 4.9–9.4 and 1.6–10.3 g/L, respectively (levulinic acid 0.3–0.6 g/L in contrast). It was important to note that, contrary to the primary trends shown in this figure, formic acid formation decreased to varied extent when temperature was raised up. As for the case of furans shown in Fig. 2c, the overall desirability was strongly affected at low lime loadings. In fact, the inhibitory effect of furans was mainly attributed to HMF due to its high concentration in the hydrolysate (up to 3.1 g/L). HMF formation accelerated at high temperatures, especially with low lime loading. However, interestingly, HMF accumulation reduced with more lime used in the pretreatment but leveled off at high lime loading. Putting three plots together in Fig. 2, we can conclude that similarly as in hemicellulose hydrolysis under acid conditions, during lime pretreatment, raising temperature could facilitate cellulose hydrolysis, but high temperature noticeably further degraded glucose to other by-

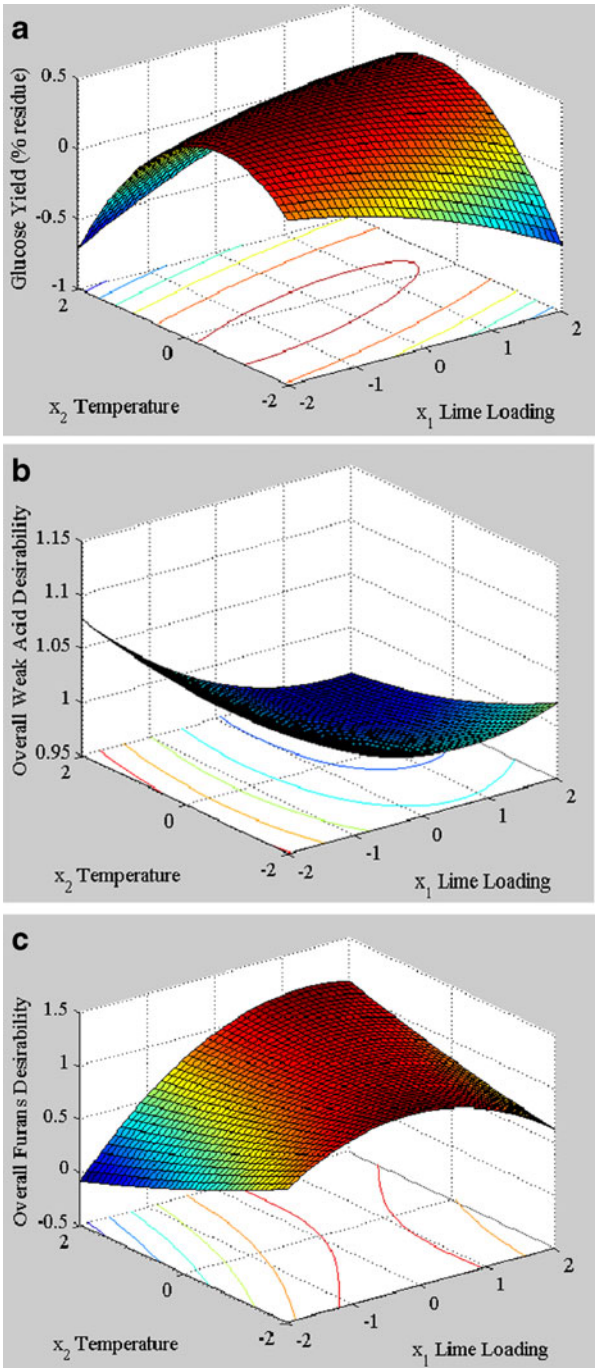


Fig. 2 Response surfaces and contour plots in second-stage alkaline pretreatment as a function of lime loading (x_1) and temperature (x_2). **a** Glucose yield; **b** overall weak acid desirability integrating acetic, formic, and levulinic acids; **c** overall furans desirability integrating furfural and HMF

products. However, lime could slow down the latter unwanted side reaction to certain extent. Besides, the remaining hemicellulose after acid pretreatment would not only be hydrolyzed to xylose but mostly further down to formic acid.

Primary phenolic compounds generated through lime pretreatment were listed in Table 2 along with their concentrations. It has been found that the phenols present in hydrolysates were strongly dependent on the pretreatment type [26]. For that matter, occurrence of different phenols in lime-treated hydrolysates was noted in comparison with previous acidic hydrolysates. As a result, all phenols were produced through acid pretreatment, but ferulic acid was found during lime pretreatment. Furthermore, lime pretreatment generated some unique phenols like syringol and methylhydroquinone. Among the detected phenols, vanillin and syringol were the most abundant. In addition, most phenols through lime pretreatment were lignin blocks with more complicated structure, which suggested that alkaline pretreatment led to incomplete lignin breakdown compared with acid pretreatment. We can also learn from Table 2 that generally higher operational severities could induce more phenols production.

It has been found that high glucose recovery can be obtained under two conditions during alkaline pretreatment, either long pretreatment time and low temperature, or high temperature for a short time [32]. Previously, alkaline pretreatment was commonly employed at lower temperatures (50–130 °C) for extended times on the order of hours, to avoid the great loss of hemicellulose. In this work, most hemicellulose was removed in the prior acid stage, so lime pretreatment can be explored at temperatures above 170 °C with much shortened reaction time, more favorable from an economic perspective. In fact, the applied temperatures were even higher than the previous stage to attain elevated severities for enhanced biomass susceptibility to enzymatic hydrolysis. Similar as in dilute acid pretreatment, little was known about the effects of pretreatment conditions on lime pretreatment performance especially their interactive effects [16, 33]. Other than that, since acid pretreatment was applied ahead, different profiles after lime pretreatment could be expected in this case. Indeed, small amount of lime was necessary, and there was different effect of applied temperature at high levels on glucose recovery. Normally, a lime loading of up to 0.1 g/g of dry biomass was recommended in terms of high sugar recovery [32, 34], but the amount needed was reduced to as low as 0.01 g/g in the current study. Apparently, lime appeared to be more active at elevated temperature to disrupt the cellulose crystallinity and increase the biomass porosity. Meanwhile, on the flip side, enhanced lime activity also meant calcium ions could easily interact with lignin and carbohydrates with high affinity and thus impact glucose release [35], implying redundant lime addition was of no benefit. It can be demonstrated by the noticeable decline of glucose yield with increased lime loading at low temperatures in Fig. 2a. In addition, at elevated temperatures, significant drop of glucose yield occurred from its own degradation, which was not observed at mild temperatures. Stripping off most hemicellulose and significant alternation of lignocellulose structure prior to alkaline stage would also cause the cellulose more sensitive to the temperature.

For the optimization of lime pretreatment, when taking account of the four major groups of products (sugars as glucose, weak acids, furans, and phenols), a compromise was made, and the best conditions were located at 0.024 g/g biomass of lime loading and 202 °C. Under these conditions, glucose yield was among the highest (78.2 % theoretical) with generally lower acetic acid, furan, and phenol production, as discussed in the following section.

Fates of Lignocellulose Components

To provide perspective into the process mechanism for ACAL pretreatment, a holistic view of the fates of primary degradation products and their distribution in the system would be necessary. Therefore, sugar degradation products from cellulose and hemicellulose were measured and presented in Fig. 3. In addition to ACAL, single-stage dilute sulfuric acid and

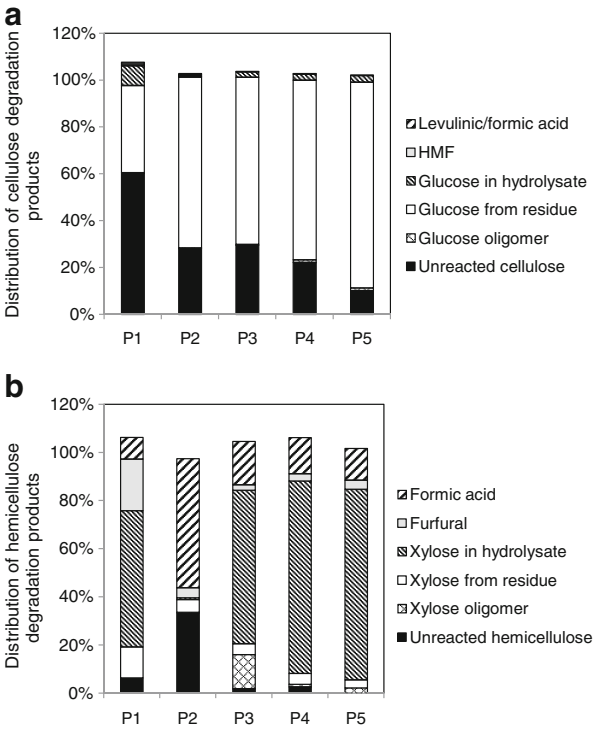


Fig. 3 Sugar degradation products from cellulose (**a**) and hemicellulose (**b**) resulting from pretreatment schemes described in Table 3

lime pretreatments were carried out individually under their own best conditions, for comparison purpose. Moreover, our previous study [20] showed the combined biomimetic and inorganic acids could substantially improve the hemicellulose hydrolysis and recover more xylose. Thus, the combined acid catalysts with two representative biomimetic acids, TFA and MA, were introduced into ACAL to assess the pretreatment efficiency of the integrated process. All the tested pretreatment schemes were described in Table 3.

For cellulose degradation products, as can be observed from Fig. 3a, single acid pretreatment (P1) left considerably more cellulose intact than the other pretreatment schemes. It verified that acid catalysts were not efficient in glucose recovery. In contrast, ACAL (P3) led to nearly the same profile of cellulose degradation products as single alkaline pretreatment (P2). Recovered glucose mainly came from the treated residue after enzymatic hydrolysis, indicating the second alkaline stage played the key role for glucose recovery. When combined acid catalysts were adopted in ACAL (P4/P5), glucose recovery was further

Table 3 Operational conditions of various pretreatment schemes for comparison

Pretreatment schemes	Acid stage	Alkaline stage
P1	1.0 wt% H_2SO_4, 170 °C, 15 min	
P2	0.024 g/g $Ca(OH)_2$, 202 °C, 30 min	
P3	0.73 wt% H_2SO_4, 150 °C, 6 min	0.024 g/g $Ca(OH)_2$, 202 °C, 30 min
P4	0.375 wt% H_2SO_4+4 mg/L TFA, 150 °C, 6 min	0.024 g/g $Ca(OH)_2$, 202 °C, 30 min
P5	0.548 wt% H_2SO_4+15.6 g/L MA, 150 °C, 6 min	0.024 g/g $Ca(OH)_2$, 202 °C, 30 min

improved by 8–23 %. On the other hand, for hemicellulose degradation products shown in Fig. 3b, the profile of ACAL was similar to single acid pretreatment instead. As was reported previously, lime pretreatment would be ineffective for hemicellulose decomposition [8]. But unexpectedly here, nearly all the degraded hemicellulose went directly down to furfural. It appeared that lime was more efficient in catalyzing xylose degradation than hemicellulose decomposition, although further work was required for verification. On the contrary, single acid pretreatment could convert most hemicellulose into xylose. However, since an elevated severity was applied as not to lose much glucose, a fair amount of xylose was inevitably degraded to furfural at the same time. ACAL could achieve efficient hemicellulose decomposition, primarily in the acid stage. Meanwhile, the separate pretreatments in ACAL allowed a low severity application in the acid stage and ensured higher xylose recovery. Similarly as for cellulose profile, introduction of combined acid catalysts in ACAL could obtain higher xylose yield through thorough conversion of oligomeric xylose.

Along with degradation of sugar polymers, lignin degradation during pretreatment was examined, and the individual and total phenols under various pretreatment schemes were summarized and compared in Table 4. Single lime pretreatment caused substantial accumulation of total phenols, higher than single acid pretreatment by over 30 %, conceivably originating from great presence of unique complex lignin-derived intermediates such as syringol and hydrocinnamic acid. Compared with both single-stage pretreatments (P1 & P2), ACAL led to significantly reduced accumulation of most phenolic compounds. Since the severity was lowered in the acid stage, much less phenols were generated during acid pretreatment. In the meantime, it was also interesting to note that lignin with most

Table 4 Major products in the prehydrolysates under various pretreatment schemes

Pretreatment schemes		P1	P2	P3	P4	P5
Weak acids (g/L)	Acetic acid	8.5	11.2	5.7 (81 %)	7.3 (80 %)	8.6 (79 %)
	Formic acid	1.8	9.4	1.3 (23 %)	1.2 (20 %)	1.1 (25 %)
	Levulinic acid	0.6	0.4	0.1	0.1	0.2
Furans (g/L)	Furfural	7.7	1.5	0.9 (97 %)	1.1 (96 %)	1.5 (88 %)
	HMF	0.5	0.4	0.1	0.1	0.1
Phenols (mg/L)	Total phenols (g/L)	4.1	5.6	3.0 (29 %)	3.9 (18 %)	2.9 (24 %)
	P-Hydroxybenzaldehyde	18.8	9.2	9.7	3.3	2.3
	P-Hydroxybenzoic acid	13.7	5.3	2.8	1.1	0.9
	Vanillin	76.0	7.4	21.0	7.3	6.4
	Vanillic acid	37.4	4.9	4.6	1.8	1.8
	Syringaldehyde	34.3	3.5	13.2	5.6	5.1
	Syringic acid	17.1	3.0	2.5	1.2	0.9
	P-Coumaric acid	102.0	3.2	66.7	38.0	27.2
	Ferulic acid	46.1	5.3	11.0	5.0	4.1
	Syringol		9.1	0.7	1.2	0.4
	3-Vanillyl propanol		3.2	0.4	0.5	0.1
	Hydrocinnamic acid		48.6	0.7	0.2	0.2
	Homovanillic acid		6.6	0.3	0.4	0.1

Percentage of products derived from the hydrolysates in alkaline stage was shown in the brackets; concentrations of syringol down to homovanillic acid were shown in ratios to 1.67 mg/L phthalic acid; 1. 1.67 mg/L phthalic acid equals 1.5–4.0 mg/L phenol depending on the phenol type

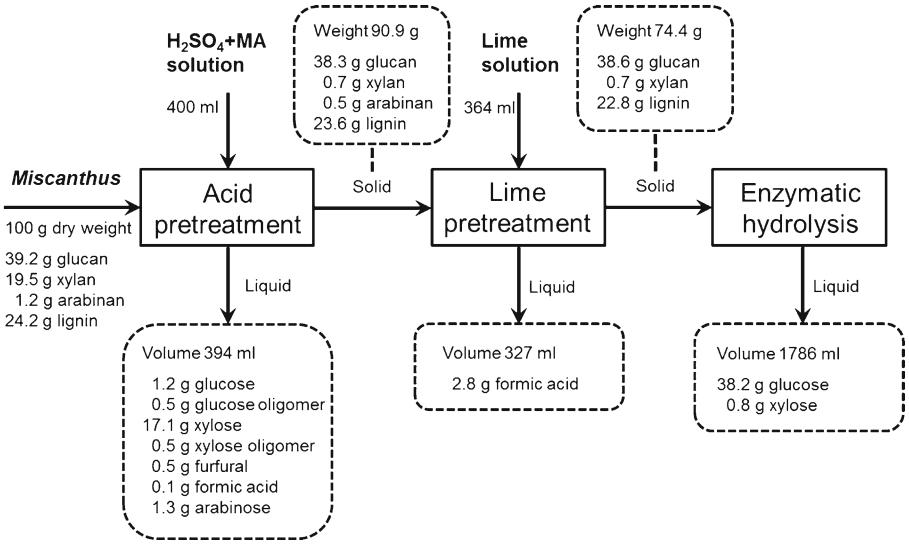


Fig. 4 Material balance flow diagram of two-stage pretreatment process with combined H_2SO_4 and maleic acid in the acid stage

hemicellulose removed appeared to be more stable during lime pretreatment. Among generated phenols, p-coumaric acid and vanillin were present with the highest concentration. When combined acids were introduced in ACAL, phenol production was further inhibited. It seemed delignification was partly avoided, as can be seen as another positive synergistic effect between H_2SO_4 and biomimetic acids. Further inquiry was needed to clarity the mechanism of lignin degradation prevention in the two-stage processes.

The degradation of lignocellulose components were also examined in terms of material flow balance throughout the entire process. Figure 4 illustrated the ACAL process with combined MA pretreatment. Mass balance was calculated in the way suggested by Percival Zhang et al. [36]. Most xylan in the biomass was efficiently removed in the form of xylose in the acid stage with low degree of severity. By contrast, the following lime pretreatment managed to enhance the glucose susceptibility to enzymatic hydrolysis and obtained high recovery rate. However, lignin mostly stayed in the biomass in both stages but did not exert great adverse effect on hydrolysis and fermentation steps. Delignification was not observed, especially in alkaline stage. One plausible explanation was that calcium ions extensively crosslinked lignin molecules and thus prevented lignin solubilization. Meanwhile, calcium also crosslinked carbohydrates, protecting them from unwanted degradations. Therefore, the situation with high lignin content was also able to avoid poor enzymatic digestibility only if the biomass porosities were effectively improved [37]. From the illustrated material flow in the figure, in total 18.4 g xylose and 39.9 g glucose could be attained after the proposed pretreatment scheme on the basis of 100 g feedstock.

Overall Pretreatment Effectiveness

The overall performance of two-stage acidic–alkaline pretreatment was scrutinized and compared with other tested pretreatment alternatives in terms of sugar yields and ethanol yields.

As can be seen from Fig. 5, all two-stage pretreatments could achieve high yields of both glucose and xylose (at least 81 % and 68 % of theoretical individually). Glucose mainly came from solid residue through enhanced enzymatic hydrolysis while xylose from acid hydrolysate. ACAL with combined acids could further improve xylose yield up to 85 %, apparently because most oligomeric intermediates were completely hydrolyzed. Furthermore, as was pointed out previously, ACAL with combined MA pretreatment could facilitate cellulose hydrolysis with enhanced glucose recovery up to 91 %.

Finally, the sugar-enriched residues after pretreatments were enzymatically hydrolyzed and fermented by engineered *S. cerevisiae* in a single step, with the ethanol yields shown in Table 5. Throughout SSCF, pH was not controlled and decreased slightly to 4.6–4.7. Both single acid and alkaline pretreatments ended with very low

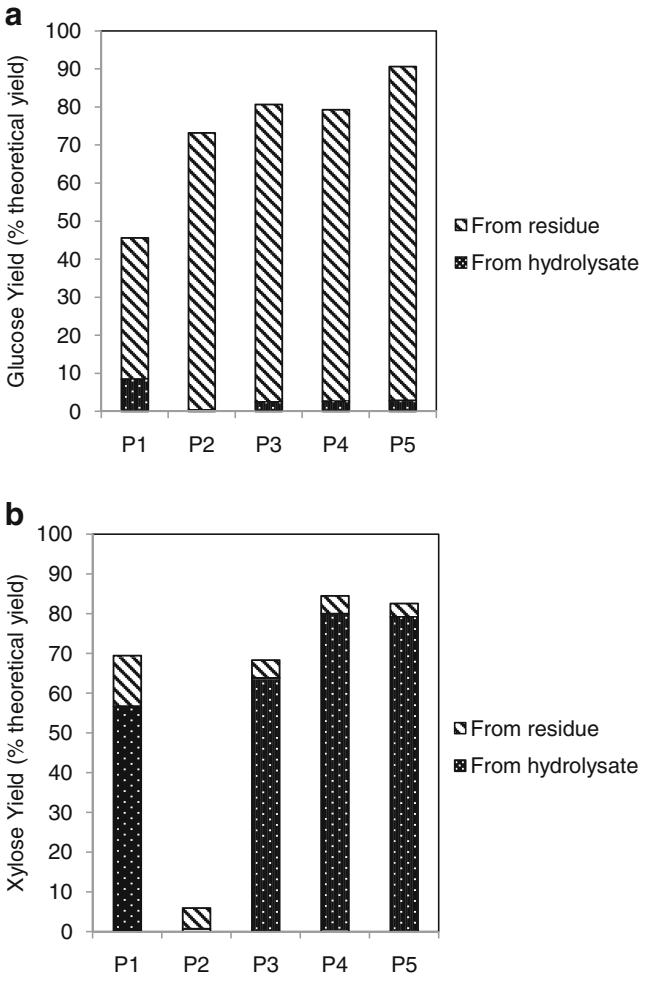


Fig. 5 Glucose (**a**) and xylose (**b**) yields under pretreatment schemes described in Table 3

Table 5 Concentrations of major compounds in the SSCF hydrolysates under various pretreatment schemes (in grams per liter)

Pretreatment schemes	P1	P2	P3	P4	P5
Glucose at 0 h	5.3	1.3	1.8	2.2	2.3
Glucose at 48 h	18.0	22.3	0.0	0.0	21.5
Glucose at 96 h	18.6	21.8	0.0	0.0	0.1
Xylose at 0 h	14.0	0.4	13.1	18.1	17.8
Xylose at 48 h	14.3	1.8	6.0	12.3	18.4
Xylose at 96 h	13.9	1.9	3.1	8.8	12.1
Ethanol at 48 h	0.2	1.0	11.0	12.9	1.6
Ethanol at 96 h	1.4	3.1	11.1	14.7	15.9
Ethanol yield (grams per gram of dry *Miscanthus*)	0.011	0.026	0.093	0.132	0.145
Ethanol yield of theoretical maximum	10 %	20 %	57 %	63 %	62 %

ethanol yield, mostly due to inefficient fermentation with only 10–20 % of theoretical ethanol yield, which was noticeably less than normal. During SSCF, the ongoing reactions ended up with steady accumulation of soluble sugars, implying the great presence of phenols and furans that significantly disturbed the fermentation process but enzymatic hydrolysis. However, acetate formed through SSCF should not be counted for the inhibition since the occurring level by these single-stage pretreatments was even lower than that by two-stage processes. In contrast, for sequential pretreatments schemes, ethanol yields were appreciably higher (57–63 %) which reflected their benefit of less inhibitory by-products induction. In terms of substrate uptake, glucose was rapidly consumed at the beginning, whereas overall xylose uptake rate was relatively low (less than 52 %). It indicated the genetic modified yeast still needed further improvement to withhold harsh fermentation environment and reach desirable xylose consumption rate. Additionally, the considerable accumulation of acetate through SSCF might also contribute a lot to the perceived inhibitory effects. Acetate concentration in the hydrolysates after two-stage pretreatments was raised from initial 2.9–3.6 up to 6.8–10.5 g/L. Among tested two-stage process, the scheme applying combined MA catalysts achieved the highest ethanol yield of 15.9 g/L, corresponding to a high yield of total reducing sugar of 65.5 g/L.

Conclusions

A pretreatment method with successive acidic and alkaline stages (ACAL pretreatment) was developed. In contrast to single-stage pretreatments which efficiently obtain one sugar product alone, two-stage process could achieve high recovery of both glucose (>80 %) and xylose (>70 %). Xylose was mainly recovered from acid stage, while glucose was secured through lime pretreatment. Meanwhile, production of weak acids, furans, and phenols were remarkably reduced. The best performance could be arrived at medium severities in the acid stage and high severities in the alkaline stage. Integration of combined acid catalysts and ACAL could further improve both sugar yields and reduce primary by-products formation, with ethanol yield of up to 0.145 g/g *Miscanthus*.

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