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Reducing acid in dilute acid pretreatment and the impact on enzymatic saccharification

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Abstract Dilute acid pretreatment is a leading pretreatment technology for biomass to ethanol conversion due to the comparatively low chemical cost and effective hemicellulose solubilization. The conventional dilute acid pretreatment processes use relatively large quantities of sulfuric acid and require alkali for pH adjustment afterwards. Significant amounts of sulfate salts are generated as by-products, which have to be properly treated before disposal. Wastewater treatment is an expensive, yet indispensable part of commercial level biomass-to-ethanol plants. Therefore, reducing acid use to the lowest level possible would be of great interest to the emerging biomass-to-ethanol industry. In this study, a dilute acid pretreatment process was developed for the pretreatment of corn stover. The pretreatment was conducted at lower acid levels than the conventional process reported in the literature while using longer residence times. The study indicates that a 50% reduction in acid consumption can be achieved without compromising pretreatment efficiency when the pretreatment time was extended from 1–5 min to 15–20 min. To avoid undesirable sugar degradation and inhibitor generation, temperatures should be controlled below 170°C. When the sulfuric acid-to-lignocellulosic biomass ratio was kept at 0.025 g acid/g dry biomass, a cellulose-to-glucose conversion of 72.7% can be achieved at an enzyme loading of 0.016 g/g corn stover. It was also found that acid loading based on total solids (g acid/g dry biomass) governs the pretreatment efficiency rather than the acid concentration (g acid/g pretreatment liquid). While the acid loading on lignocellulosic biomass may be achieved through various combinations of solids loading and acid concentration in the pretreatment step, this work shows that it is unlikely to reduce acid use without undermining pretreatment efficiency simply by increasing the solid content in pretreatment reactors, therefore acid loading on biomass is indicated to be the key factor in effective dilute acid pretreatment.

Keywords Corn stover · Cellulase · Hemicellulase · Pretreatment · Hydrolysis

Introduction

Lignocellulosic biomass represents the world's largest renewable carbohydrate reserve and has the potential to become a major source of fermentable sugars for the production of bioethanol. It has been estimated that in the US alone, more than 1 billion tons per year of biomass could be sustainably harvested as agricultural and forest residues, which has the potential to replace as much as 30% of total US gasoline consumption [23]. Due to the complexity and recalcitrance of lignocellulosic feedstocks, pretreatment is required to remove lignin and/or disrupt the structure of crystalline cellulose to increase accessibility for glycolytic enzymes [21]. In most cases, acid or alkali is used to achieve higher pretreatment efficiency as compared to pretreatment with water only as catalyst. Additional chemical input is also required after pretreatment to bring the pH to within the optimal range in which most hydrolytic enzymes function. As a result, the hydrolysate always

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contains salts (ammonium or sodium sulfate) at high concentrations, which are detrimental to yeast fermentation and expensive to dispose of. Wastewater treatment is an indispensable element in the biomass to ethanol process. However, in the literature, biomass waste management, including recycling, remediation, and disposal, is largely overlooked, partially because the second-generation biofuels industry is still in its infancy.

Whether an acid or alkaline process is implemented, the waste streams generated in bioethanol production have to be treated before recycling to the process or releasing to the environment. For both processes, wastewater treatment is expensive and complex. A recent technical report from the National Renewable Energy Laboratory provided the first wastewater treatment layout for a lignocellulosic ethanol process [12]. It involves anaerobic digestion to remove sulfate ions, ammonium ion nitrification to nitric acid, combustion of biogas containing hydrogen sulfide, neutralization of nitric acid and SO_2 from flue gas, and further removal of salts by reverse osmosis. Out of the proposed $232 million total equipment cost, 21.3 and 28.4% are allocated to wastewater treatment and boiler/turbogenerator, respectively. The costs are significantly higher than those for pretreatment, which is widely perceived as the most expensive capital investment in the second-generation bioethanol plant [12].

Alkaline pretreatment is another process that has been intensively studied. To achieve sufficient delignification and high cellulose conversion, chemical input is usually in the range of 0.075–0.5 g alkali/g of dry biomass [15, 18] depending on the substrate composition as well as other pretreatment conditions. It is possible to recover and regenerate chemicals through well-established lime kiln technology. However, the capital cost of the chemical recovery process (recovery boiler, evaporator, and lime kiln) largely limits the wide application of alkaline pretreatment technology [3]. Unless an existing paper mill is re-purposed as a lignocellulosic bioethanol plant, the alkaline process is unlikely to be economically feasible with current technology [13].

Regardless of the pretreatment chemistry, low chemical input is always desirable from both process control and process economic points of view [29]. The objective of this study was to determine the feasibility of reducing chemical usage in biomass pretreatment by utilizing longer pretreatment residence times and supplementing the hydrolysis process with improved cellulase and hemicellulase enzymes. Corn stover was selected as a model feedstock and a series of dilute acid pretreatments were conducted using combinations of relatively low acid loadings and temperatures. The efficiency of pretreatment was then evaluated by total sugar release from enzymatic hydrolysis of the pretreated substrates.

Materials and methods

Feedstock collection and preparation

Corn stover was obtained from the Midwest region of the US. During the harvest of grain, the residue (consisting of leaves, stalks, and husks) over 18 inches above the ground was collected. The corn stover residue was then milled to a 6-mm particle size using a Thomas Wiley mill. The moisture content of the corn stover was about 10%. Compositional analysis of the raw corn stover shows that it contains 38.0% glucan, 20.2% xylan, and 19.5% acid insoluble lignin on a dry basis.

Enzymes

Two cellulases mixtures were used in this study. Cellic® CTec2 from *Trichoderma reesei* was obtained from Novozymes North America (Franklinton, NC). In addition, an experimental enzyme cocktail containing both cellulase and hemicellulase activities was obtained from Novozymes, Inc. (Davis, CA). For comparison purposes, the experimental enzyme mixture was formulated to match the protein concentrations of Cellic® CTec2. Cellic® CTec2 and the experimental enzyme cocktail were stored at 4 and −30°C, respectively, until needed for hydrolysis of pretreated corn stover.

Dilute acid pretreatment

Dilute acid pretreatment was performed in two different experimental setups: a sandbath reactor, which was used for small-scale biomass pretreatment, and a Parr reactor that allowed for generation of larger quantities of material for evaluation.

Sandbath pretreatment

To determine whether the acid concentration in the aqueous phase or the acid loading on a dry corn stover basis determines the pretreatment efficiency, dilute acid pretreatment of corn stover at different total solid levels was conducted in closed tubular reactors that were heated with a Techne precision fluidized sandbath (Grants Pass, Oregon). The main body of the tubular reactor ($1/2'' \times 6''$) was constructed out of Monel alloy and capped with 316 stainless-steel caps on both ends of the reactor during pretreatment. Before loading the reactors, 20 g of dry corn stover was mixed with sulfuric acid to achieve the desired total solid loading and acid loading (Table 1). To ensure even distribution of acid, the mixtures of corn stover and acid were incubated in sealed containers at room temperature for 24 h before loading into tube reactors. Approximately 13 g of

Table 1 Dilute acid pretreatment of corn stover in sandbath reactor

Sample ID	Pretreatment total solid (%)	Acid solution conc. (g acid/g pretreatment liquor)	Biomass acid loading (g acid/g dry corn stover)
1	15	0.0051	0.0286
2	20	0.0051	0.0201
3	25	0.0051	0.0150
4	30	0.0051	0.0116
5	15	0.0051	0.0286
6	20	0.0071	0.0286
7	25	0.0095	0.0286
8	30	0.0122	0.0286

pre-soaked corn stover was loaded into each tubular reactor. The reactors were then sealed with stainless-steel caps and pretreated at 170°C for a residence time of 13 min. Pretreatment temperature was monitored with a thermocouple inserted through one of the reactor caps. The heat-up time to reach target temperature (170°C) was approximately 5 min. The pretreatment time was set as zero once the center of the reactor reached the target temperature. No agitation of corn stover substrate was provided in the tubular reactors. After pretreatment, tube reactors were immediately quenched in an ice bath for rapid cooling. Corn stover was recovered and stored at 4°C.

Pretreatment with Parr reactor

Other dilute acid pretreatments were conducted in a high-pressure stirred reactor with direct steam injection (Parr Instrument Company, Moline, IL). Approximately 200 g of dry corn stover was thoroughly mixed with the desired amount of sulfuric acid to reach a moisture content of approximately 40% (w/w). The acid impregnated corn stover was then loaded into the reactor vessel. Unlike sandbath reactor in which the reactors was heated by fluidized sand, Parr reactor allows direct steam injection to the reactor chamber to rapidly reach pretreatment temperature within 10 s. To avoid excessive condensation of steam during pretreatment, an electric heating mantle with PID control surrounding the cylindrical body preheats the cold reactor body before steam injection and can continue to provide heat during the pretreatment to lessen the amount of steam required to maintain adequate temperature. During pretreatment, the reactor contents were constantly agitated with built-in scraping impellers inside the reactor chamber to ensure thorough mixing of chemicals and feedstock. Upon completion of the pretreatment, corn stover was quickly discharged to a stainless-steel cyclone through the discharge ball valve at the bottom of the reactor. Pretreated corn stover was collected and stored at 4°C.

Enzymatic hydrolysis

Batch enzymatic hydrolysis was performed in 50-ml Nalgene polycarbonate centrifuge tubes (Thermo Scientific, Pittsburgh, PA). Pretreated corn stover was mixed with 50 mM sodium acetate buffer (pH 5.0) supplemented with enzymes as well as 2.5 mg/l penicillin to prevent microbial growth. The final total solid concentration was 10% (w/w) for sandbath pretreated corn stover and 20% (w/w) for that generated in the Parr reactor. The selection of hydrolysis total solids is dependent on the total solid level of pretreated biomass. The reaction mixtures (20 g) were agitated in a hybridization incubator (Combi-D24, FINEPCR®, Yang-Chung, Seoul, Korea) at 50°C for 120 h. Dose profile curves were constructed using 0.011, 0.016, 0.021 g Cellic® CTec2/g corn stover to evaluate pretreatment efficiency. At the end of hydrolysis, 500 µl of hydrolysate was transferred to a Costar Spin-X centrifuge filter tube (Cole-Parmer, Vernon Hills, IL) and filtered through a 0.2-µm nylon filter during centrifugation (14,000 rpm, 5 min). Supernatant was acidified with 5 µl of 40% (w/v) sulfuric acid to deactivate residual enzyme activity and analyzed by HPLC for sugar concentrations.

Feedstock compositional analysis and sugar analysis

Total solid content, fraction of insoluble solid, structural carbohydrate and lignin content of raw corn stover and pretreated corn stover were analyzed by following the standard laboratory analytical procedures (LAP) developed by the National Renewable Energy Laboratory [27, 28]. Monomeric sugars were measured by an Agilent 1200 series modular HPLC with quaternary pump, thermostatted autosampler, temperature-controlled column compartment, and refractive index detector (Santa Clara, CA). Separation was performed at 80°C with a Bio-Rad Aminex HPX-87P column, 300 × 7.8 mm (Bio-Rad No. 125-0098) with anion/cation Micro-Guard De-Ashing cartridges (Bio-Rad No. 125-0118) and Micro-Guard Carbo-P cartridges (Bio-Rad No. 125-0119). The separation was run isocratically with a mobile phase of deionized water (≥18.2 MΩ-cm) at a flow rate of 0.6 ml/min. The concentrations of glucose, mannose, xylose, galactose, and arabinose were measured in each sample on a peak area basis by refractive index with an external calibration set made up of the same compounds.

Sugars released from hydrolysis of pretreated corn stover was analyzed with an HPLC system (1200 Series LC System, Agilent Technologies Inc., Palo Alto, CA) equipped with a Rezex ROA-Organic acid H^+ column (8%)

(7.8 × 300 mm) (Phenomenex Inc., Torrance, CA), 0.2 μm in line filter, an automated sampler, a gradient pump, and a refractive index detector. The mobile phase used was 5 mM sulfuric acid at a flow rate of 0.9 ml/min. Monomeric sugars at concentrations of 0, 10, 30, and 50 mg/l were used as standards. The overall glucan/xylan conversions from pretreatment and hydrolysis were calculated based on sugars in enzyme hydrolysis supernatant and biomass composition of the raw feedstock using a method similar to that published by Zhu et al. [33].

Results and discussion

Effect of acid solution concentration and biomass acid loading

Dilute acid pretreatment is one of the most intensively investigated pretreatment methods for conversion of lignocellulosic biomass to ethanol. It enhances feedstock digestibility mainly through the dissolution of the more labile hemicellulose fraction and possibly by partial hydrolysis of the recalcitrant cellulose fraction [26]. The efficiency of dilute acid pretreatment is dependent on the combined effect of three parameters: pretreatment temperature, residence time, and acid charge. Acid concentration in pretreatment solution is widely used as an indicator of acid strength [17, 32]. However, depending on the total solid loading in pretreatment, the acid charge on a per unit feedstock basis may vary significantly even when acid solution concentration is constant. Whether acid solution concentration (g acid/g pretreatment liquor) or biomass acid loading (g acid/g biomass) is the more dominant factor in determining biomass digestibility is not very clear in the literature.

To determine which parameter is the determining factor for substrate digestibility, dilute acid pretreatment of corn stover was conducted at total solid loadings of 15–30% by 5% increments. Samples 1–4 had constant nominal acid solution concentration while samples 5–8 had constant biomass acid loading (Table 1). The actual sulfuric acid concentration in the liquid may be lower than the nominal concentration as the minerals contained in the raw stover can neutralize acid and increase the acid demand [6, 8]. Figure 1 presents the concentrations of monomeric sugars and acetate in pretreatment liquors separated from pretreated corn stover under two different scenarios, pretreatment with constant nominal acid solution concentration (Fig. 1a) and pretreatment with constant nominal acid loading per unit dry biomass (Fig. 1b). All pretreated corn stover samples were diluted to 15% total solids loading for comparison purposes. Biomass acid loading was also calculated and plotted on the secondary Y-axis. In Fig. 1a, the nominal acid concentration was maintained at a constant level of 0.51% while the biomass acid loading decreased from 2.86 to 1.16% as the total solid level in pretreatment increased from 15 to 30%. It was clear that the concentrations of glucose, xylose, and acetate in the pretreatment liquor decreased as the pretreatment total solids level increased, indicating that they are more closely correlated with biomass acid loading than acid solution concentration. This observation is also supported by the data in Fig. 1b, where the concentrations of monomeric sugars and acetate remained relatively stable when the biomass acid loading was held constant.

Pretreated corn stover was then neutralized to pH 5 with sodium hydroxide. To reduce the processing complexity and cost, post-treatment of pretreated corn stover such as solid–liquid separation or washing the substrate to remove soluble inhibitors was not performed. The whole pretreated biomass slurry was hydrolyzed with 0.016 g Cellic® CTec2/g raw corn stover for 120 h at 10% total solids loading. Significant difference in composition of pretreated corn stover slurry (glucan/xylan content, monomeric, oligomeric sugar, and soluble inhibitors concentrations) was expected after pretreatment of various conditions. In this study, enzyme was therefore chosen to be dosed based

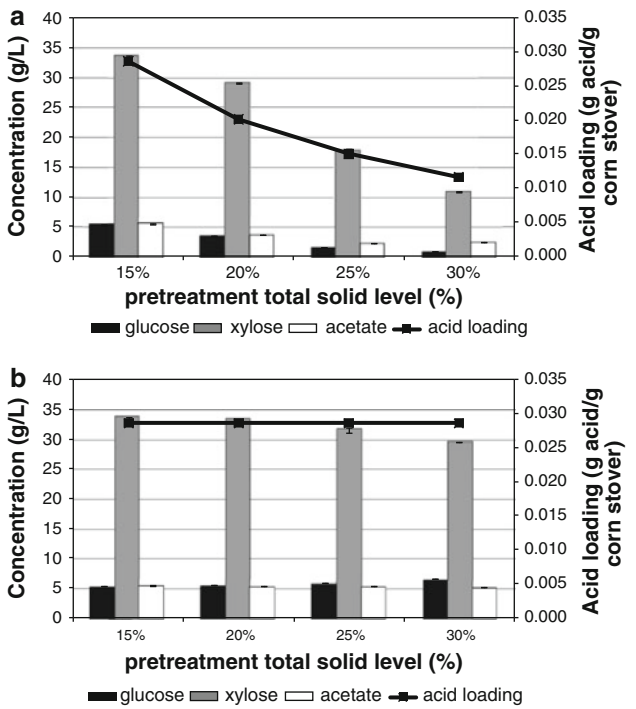

Fig. 1 Effect of acid loading on monomeric sugars and acetate concentrations in pretreatment liquor when **a** acid solution concentration was maintained at 0.0051 g acid/g pretreatment liquor and **b** when biomass acid loading was maintained at 0.0286 g acid/g corn stover. All pretreatment liquors were diluted to 15% total solids level for comparison purposes

on the weight of raw corn stover for comparison purposes. Concentrations of soluble sugars from both pretreatment and hydrolysis steps were used to calculate the overall glucan/xylan conversions from raw feedstock using a method published by Zhu et al. [33]. Figure 2 shows glucose and xylose yields from hydrolysis of corn stover pretreated with constant nominal acid solution concentration (Fig. 2a) and with constant nominal biomass acid loading (Fig. 2b). Essentially, the enzymatic digestibility of the corn stover has a better correlation with biomass acid loading than with acid solution concentration. Glucose/xylose concentrations in the hydrolysate increased as the biomass acid loading increased (Fig. 2a) and remained relatively constant when it was maintained at 2.86% (Fig. 2b). When pretreatment was conducted at high total solid levels, e.g., 30%, xylose concentration in the pretreatment liquor as well as in the hydrolysate is comparatively lower (Figs 1b, 2b). This trend may be attributed to the higher degree of xylose degradation when moisture content is low. During acid pretreatment, protonation of the xylose hydroxyl groups on the sugar ring has been shown to be a rate-limiting step in sugar degradation to furfural and/or other one carbon (formic acid) and four carbon products [9]. The presence of water molecules can significantly slow down the degradation of xylose because the protons can be quickly transferred away from the xylose hydroxyl group to other water molecules due to the strong hydrogen bonding interactions between the water molecules and due to high proton mobility [24].

Figure 3 presents substrate accessibility to enzymes, which was measured by overdosing two sets of substrates with 0.13 g Cellic® CTec2/g corn stover. Similarly, the results indicated that enzymatic accessibility depends more on biomass acid loading than acid solution concentration.

When lignocellulosic materials are subjected to an acidic environment, acid-catalyzed hydrolysis of glycosidic linkages in polysaccharides, cleavage of α- and β- aryl ether bonds in lignin, and possible splitting of the lignin-carbohydrate bonds are the primary reactions [19]. In these reactions, acid functions mainly as a catalyst to first protonate glycosidic oxygen and/or ether oxygen moieties. The reaction then proceeds into a rate-limiting step in which protonated molecules are converted into intermediates which could be carbonium-oxonium ions formed during cleavage of polysaccharides and benzylic carbonium ion-quinone-methide resulting from the depolymerization of lignin. After their reaction with water to produce monomeric sugars or other degradation products, a proton is released [2, 19]. Despite the fact that a proton is not consumed in these reactions, the rate of hydrolysis still depends on the concentration of the protonated molecules formed through direct interaction between acid and lignocellulose since their conformation to intermediates is the

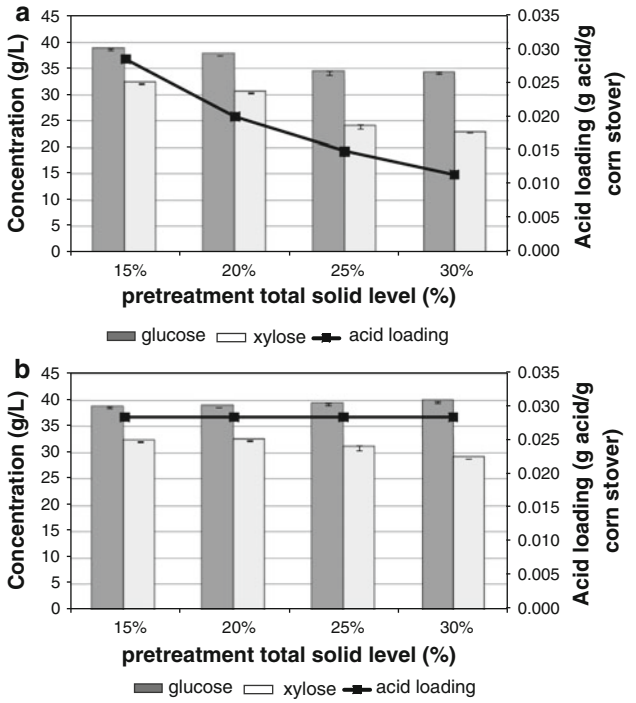

Fig. 2 Effect of acid loading on hydrolysis of corn stover pretreated with **a** 0.0051 g acid/g pretreatment liquor and **b** 0.0286 g acid/g corn stover. Hydrolysis of pretreated corn stover was conducted at 10% total solids level

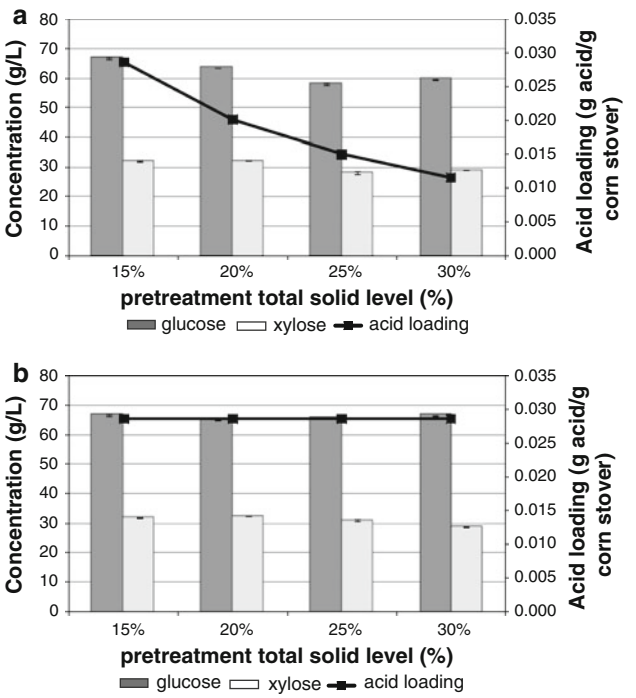

Fig. 3 Total enzyme accessibility of corn stover pretreated with **a** 0.0051 g acid/g pretreatment liquor and **b** 0.0286 g acid/g corn stover. Hydrolysis of pretreated corn stover was conducted at 10% total solids level

rate-controlling step. When pretreatment time is constant, higher acid concentration on dry biomass means a higher rate of protonation, therefore a faster reaction rate and higher product yields [4]. Ash is widely present in lignocellulosic materials, which consists mainly of calcium, potassium, magnesium, and phosphorus. This inorganic material has neutralizing ability that can consume significant amounts of acid which would otherwise be utilized for depolymerization of biomass. Since the acid demand from ash is positively correlated with the level of biomass dry matter, acid consumption resulting from the neutralization of ash increases when pretreatment total solid loading increases. It is therefore not surprising that acid loading per unit of dry biomass plays a more important role in determining substrate digestibility than does acid solution concentration. Based on this study, it can also be concluded that acid reduction is unlikely to be successful simply by increasing the solid loading in the pretreatment reactor.

Pretreatment optimization for acid reduction

Composition of pretreated corn stover

Studies in the literature on dilute acid pretreatment often targeted almost complete hemicellulose removal by utilizing relatively high acid dosages (0.02–0.05 [25], 0.07–0.30 [5], and 0.05–0.11 g acid/g biomass [8]). In this work, acid loading in pretreatment was maintained in a relatively low range (0.01–0.025 g acid/g biomass) in order to reduce salt accumulation in the hydrolysate (Table 2). Previous pretreatment optimization work showed that maximum glucose plus xylose yield was obtained when corn stover was pretreated with 0.05 g acid/g corn stover at 190°C for 1 min, which is the same as what has been reported in the literature [25]. This condition was therefore used in this study as a baseline control to determine potential improvement of substrate digestibility as well as possible reduction in chemical consumption.

Tables 3 and 4 show the composition of the washed solid and pretreatment liquor isolated from a 20% total solid slurry. Glucose concentration in pretreatment liquor ranged from 1.4 to 7.1 g/l, which was equivalent to 1.3–6.9% conversion of glucan to glucose monomer. On the other hand, a substantial amount of xylan (11.6–67.3%) was recovered as monomeric xylose in the pretreatment liquor. These results are in agreement with previous findings that glucan is more stable than xylan in an acidic environment [20]. The distribution of monomeric sugars and oligosaccharides was also affected by pretreatment conditions. When pretreatment was conducted at 0.05 g acid/g biomass, 190°C for 1 min, the primary reaction products from hemicellulose as well as cellulose solubilization were monomeric sugars. The lack of oligosaccharides can be attributed to the high proton-to-biomass ratio where protons effectively catalyze the cleavage of glycosidic bonds to release sugar monomers. On the other hand, when biomass acid loading and pretreatment temperature decreased, depolymerization of polysaccharides was less extensive, resulting in a solid fraction with higher xylan content (Table 3) and a liquid fraction containing both sugar monomers and oligosaccharides (Table 4). All pretreated corn stover samples had low 5-HMF and furfural concentrations (Table 4), which is beneficial to the ethanol fermentation process since HMF and furfural have been reported to be strong inhibitors of *S. cerevisiae* when their concentrations exceed more than 1 and 2 g/l, respectively [22].

Enzymatic hydrolysis of pretreated corn stover

Glucan and xylan conversions from pretreatment and from the overall process (pretreatment and hydrolysis) are presented in Table 5. The experimental enzyme mixture was tested at three different doses (0.011, 0.016, and 0.022 g enzyme/g corn stover). Substrate enzyme accessibility, measured as glucose and xylose concentrations after overdosing with 0.13 g enzyme/g corn stover, is presented in Table 5.

When corn stover was pretreated with 0.01 g acid/g corn stover, glucose and xylose yields from pretreatment were the lowest compared with other conditions. It seemed that extension of pretreatment time to 60 min cannot compensate for the reduction of chemical dose. Although enzymes efficiently depolymerized soluble oligosaccharides and residual cellulose/hemicellulose, as indicated by the high conversion in hydrolysis, the overall process yields for both sugars are significantly lower than pretreatment using higher acid loading. This low sugar conversion can be

Table 2 Dilute acid pretreatment conditions for corn stover

Pretreatment run	Temp (°C)	Biomass acid loading (g acid/g corn stover)	Time (min)
1	160	0.010	60
2	160	0.015	45
3	160	0.020	30
4	160	0.025	20
5	160	0.025	10
6	170	0.010	60
7	170	0.015	45
8	170	0.020	30
9	170	0.025	20
10	170	0.025	10
11	190	0.050	1

Table 3 Composition of washed pretreated corn stover solid

Temp (°C)	Acid loading (g acid/g corn stover)	Time (min)	$f_{ts0}^{a,c}$	Composition (%)[b,c]			
				Glucan	Xylan	Galactan	Arabinan
160	0.010	60	0.70	50.3	11.0	0.6	1.1
160	0.015	45	0.70	50.8	9.7	0.6	0.8
160	0.020	30	0.66	50.4	8.7	0.9	1.1
160	0.025	20	0.67	52.0	5.8	0.6	0.8
160	0.025	10	0.67	48.6	11.1	0.8	1.1
170	0.010	60	0.70	52.1	7.9	0.6	0.9
170	0.015	45	0.68	54.3	7.6	0.6	1.1
170	0.020	30	0.69	53.0	6.1	0.3	0.8
170	0.025	20	0.68	54.0	5.2	0.3	0.9
170	0.025	10	0.66	51.6	7.1	0.5	1.2
190	0.050	1	0.64	57.4	3.3	0.0	0.0

[a] Initial mass fraction of total solids in slurry

[b] The composition of insoluble solids are based on oven-dry weight

[c] Values are expressed as averages of three replicate samples. Coefficient of variation (CV) is below 2.1%

Table 4 Analysis of pretreatment liquor (g/l)

Pretreatment conditions (temp (°C), acid (g/g), time (min))	Monomeric sugars, sugar degradation products, acetate						Oligosaccharides	
	Cellobiose	Glucose	Xylose	5-HMF	Furfural	Acetate	Glucose	Xylose
160, 0.010, 60	0.0	2.2	12.5	0.13	0.33	5.8	7.8	21.7
160, 0.015, 45	0.0	1.7	13.0	0.22	0.59	6.2	5.6	16.6
160, 0.020, 30	0.0	5.7	33.2	0.26	0.71	7.1	5.1	9.4
160, 0.025 20	3.3	7.1	38.6	0.67	0.76	7.4	3.7	4.9
160, 0.025, 10	2.6	4.4	30.3	0.07	0.19	4.4	6.4	15.9
170, 0.010, 60	0.0	2.0	7.2	0.26	0.89	7.5	7.7	26.1
170, 0.015, 45	0.0	2.6	14.0	0.25	0.63	6.0	8.0	23.9
170, 0.020, 30	2.2	5.0	27.7	0.33	1.26	7.9	7.8	16.7
170, 0.025, 20	3.7	7.9	39.7	0.29	0.91	8.1	5.8	8.2
170, 0.025, 10	1.8	3.1	18.2	0.13	0.41	6.1	8.4	20.4
190, 0.050, 1	3.0	11.5	44.4	0.20	1.51	6.7	0.4	0.7

Values are expressed as averages of two replicate samples. Liquor was separated from 20% pretreated corn stover slurry. CV is below 1.2%

attributed to the insufficient removal of xylan, which has been proven to form a physical barrier restricting access to cellulose by enzymes [14]. An increase in sugar conversion was observed with increasing biomass acid loading, suggesting that xylan removal is positively correlated with cellulose digestibility when xylose degradation to furfural is minimal [14]. This result is in agreement with previous studies conducted on agricultural residues and woody biomass [1, 7]. As the acid loading in pretreatment increased to 0.025 g acid/g corn stover, the overall sugar conversion was slightly higher than that achieved with 0.05 g acid/g corn stover. The higher sugar yield obtained with mild pretreatment can be explained as follows: (1) when biomass was treated under mild pretreatment conditions, the sugar degradation was lower, therefore the sugar potential in enzymatic hydrolysis was higher (data not shown); (2) mild pretreatment conditions resulted in less organic acids and phenolic compounds which are the primary by-products from degradation of hemicellulose and lignin [16]. These byproducts have been reported to be potent inhibitors to cellulase activities [16, 30, 31]. In other words, to reach similar glucan/xylan conversion significantly higher enzyme doses are required to overcome the negative effects caused by enzyme inhibitors. With the reduction of soluble inhibitors generated in pretreatment, the performance of enzymes can be greatly improved.

Chemical consumption

To determine the alkali consumption during pH neutralization of pretreated substrates, corn stover samples that had the highest digestibility were titrated with sodium hydroxide to pH 5. Compared to the control, which had an alkali demand of 0.054 g NaOH/g pretreated corn stover, corn stover treated with 0.025 g acid/g corn stover at 160°C for 20 min required 65% less alkali to adjust its pH to 5.0. The lower alkali requirement can be partially attributed to the reduced acid input in pretreatment. In

Table 5 Overall yields of glucose and xylose from pretreatment and hydrolysis of corn stover after 120 h hydrolysis

Pretreatment conditions (temp (°C), acid (g/g), time (min))	Pretreatment		Enzyme dose (g enzyme/g corn stover)						Enzyme accessibility	
	Glucan conversion (%)	Xylan conversion (%)	0.011		0.016		0.022		Glucan conversion (%)	Xylan conversion (%)
			Glucan conversion (%)	Xylan conversion (%)	Glucan conversion (%)	Xylan conversion (%)	Glucan conversion (%)	Xylan conversion (%)		
160, 0.010, 60	1.3	11.3	45.8	60.7	56.9	66.6	64.1	70.6	79.2	75.6
160, 0.015, 45	2.3	23.8	48.9	68.9	60.4	74.8	68.7	78.7	82.0	82.5
160, 0.020, 30	4.5	45.5	53.0	74.8	64.0	79.3	71.9	82.6	82.3	85.2
160, 0.025 20	6.9	61.5	59.8	79.3	72.7	82.7	78.8	84.7	91.5	87.6
160, 0.025, 10	2.6	28.6	51.9	68.5	59.5	72.3	64.1	75.4	73.7	80.0
170, 0.010, 60	1.9	21.4	49.5	65.3	61.0	70.8	70.3	74.4	87.1	78.3
170, 0.015, 45	2.2	27.8	56.9	72.8	64.1	74.3	73.4	76.3	81.6	80.4
170, 0.020, 30	4.6	52.8	53.9	77.5	67.3	82.1	76.6	84.3	92.4	87.3
170, 0.025, 20	6.5	65.3	56.6	82.8	70.9	86.7	79.2	89.1	93.7	90.4
170, 0.025, 10	3.8	47.9	56.7	81.0	67.7	85.8	74.0	88.4	83.2	91.3
190, 0.050, 1	11.8	78.6	53.0	83.8	66.7	84.1	72.5	85.7	81.6	84.9

Enzymatic hydrolysis was performed on 20% total solid pretreated corn stover slurry. Data is the average of duplicate samples and CV is below 1.8%

addition, the degradation of sugars and lignin in the presence of acid promotes the formation of organic acids such as acetic, formic, levulinic, tannic, and gallic acid, the amount of which depends on biomass type, concentration of lignocellulose in pretreatment, and is positively correlated with severity of pretreatment [16]. Therefore, when dilute acid pretreatment is conducted at milder conditions, the declining alkali demand from degradation products also contributes to the reduction in chemical consumption [16].

The role of hemicellulase

Corn stover pretreated with 0.01–0.025 g acid/g corn stover had a xylan content in the range of 5.2–11.1%, which is significantly higher compared to corn stover pretreated with 0.05 g acid/g corn stover (Table 3). This implies that hemicellulases should be indispensable components in hydrolysis of biomass pretreated at milder conditions. To achieve better sugar yields from substrates pretreated with lower biomass acid loading and temperature, the enzyme mixture was tailored to include xylanase and other accessory enzymes to efficiently hydrolyze the remaining xylan and xylooligomers. Xylose recovery from both pretreatment and hydrolysis of pretreated corn stover is shown in Fig. 4. Hydrolysis was conducted with 0.016 g enzyme/g corn stover at 20% total solid loading for 120 h. For all the substrates pretreated with 0.01–0.025 g acid/g corn stover, 21.2–53.5% of residual xylan was further hydrolyzed to xylose monomer by enzymes. The effect of hemicellulases is especially pronounced for the substrate pretreated with 0.01 g acid/g corn stover; although the overall xylose yield was still 15–19% lower than what was obtained from the optimal set of conditions. On the other hand, only 6.6% xylose was released from enzymatic hydrolysis of corn stover treated with 0.05 g acid/g corn stover. Maximum xylan conversion was obtained at 0.025 g acid/g corn stover, 170°C for 20 min. The total xylose concentration was equivalent to that released from corn stover pretreated with 0.05 g acid/g corn stover. These results indicated that enzymes can partially replace acid in the depolymerization and hydrolysis of hemicellulose.

In biomass-to-ethanol process, pretreatment is one of the most critical unit operations. Its importance in removing the barrier of lignin and hemicellulose, reducing cellulose crystallinity, and increasing biomass porosity has been recognized for a long time. However, the overall efficiency of the process depends on a good balance between pretreatment severity and substrate digestibility. Previous studies on biomass pretreatment generally aimed at achieving near-complete solubilization of the hemicellulose fraction of biomass by utilizing high acid concentrations and temperatures as the digestibility of the cellulose fraction is largely limited by the efficiency of cellulolytic

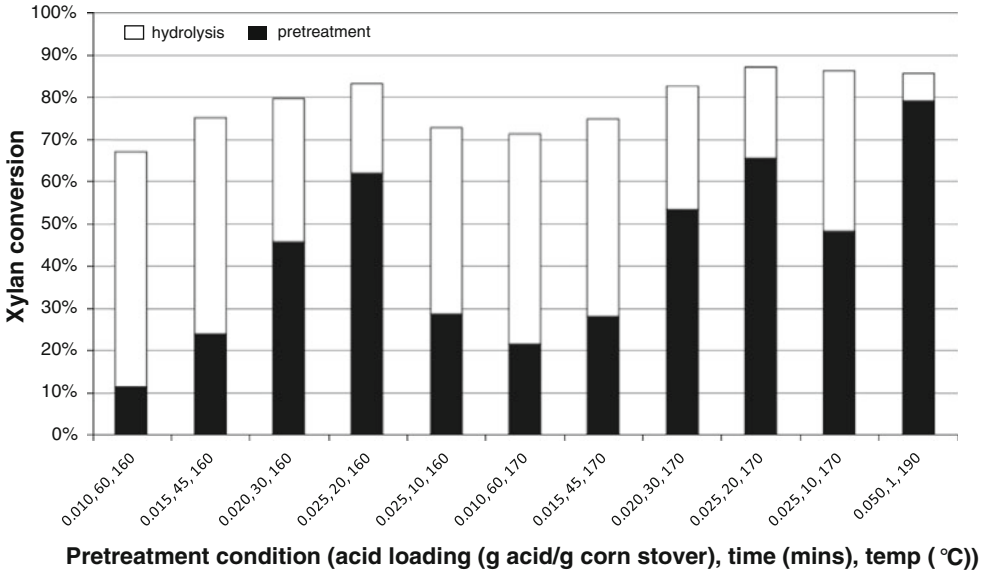

Fig. 4 Xylose conversion from pretreatment and hydrolysis of pretreated corn stover. Hydrolysis was conducted at 20% total solids level with 0.016 g experimental enzyme/g corn stover for 120 h

enzymes. Without sufficient xylan removal, previous cellulase products were unable to effectively hydrolyze cellulose. However, the cellulose, hemicellulose, and lignin fractions of biomass have very different optimum pretreatment severities for maximum recovery [11], which indicates that optimal pretreatment conditions for cellulose may cause the degradation of the hemicellulose fraction to form enzyme and/or fermentation inhibitors. At the industrial scale, it is economically challenging to remove inhibitors by washing the pretreated biomass. Therefore solubilizing hemicellulose components to fermentable sugars without further degradation is always more desirable. As the hydrolytic actions of hemicellulases are more specific towards the hemicellulose substrates without any undesirable degradation by-products, the role of hemicellulases in reducing acid use during pretreatment should be further explored.

Concluding remarks

Robust and efficient pretreatment and hydrolysis processes can be established by using the combination of less severe pretreatment conditions and more efficient enzymes to degrade both cellulose and substituted xylan. This work demonstrated that it is possible to reduce the quantity of chemicals required and also the amount of salt accumulated by replacing chemicals with hydrolytic enzymes. When the pretreatment was conducted at relatively mild conditions (lower acid loading, lower temperature, and longer time), the digestibility of the substrate was not compromised due to the use of improved enzymes and the incorporation of new enzymatic activities into the enzyme cocktail.

Reduction of chemical loading in pretreatment can benefit the overall process in many different aspects. First of all, displacing harsh chemicals with enzymatic solutions can substantially reduce chemical consumption and waste treatment/disposal costs. Moreover, it can also be beneficial for downstream fermentation as levels of fermentation inhibitors and salts are greatly reduced [10]. Since pretreatment is conducted at lower acid loading and temperature, acid corrosion of reaction vessels might also be alleviated. Due to the advances in enzymology, enzyme efficiency has been improved at an unprecedented pace. Discoveries of new cellulases and hemicellulases including depolymerizing enzymes that act on hemicellulose backbone (xylanase) and debranching enzymes that cleave glycosidic or ester linkages (ferulic acid esterases, acetyl xylan esterase) may enable pretreatment processes with very low or even no chemical as catalyzing agent in the future. With future improvements in enzyme efficiency and selectivity, it can be expected that further reduction of chemical consumption can be achieved.

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Conflict of interest The authors declare that the experiments comply with the current laws of the US. The authors also declare that they have no conflict of interest.

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