



Improvement of sugar yields from corn stover using sequential hot water pretreatment and disk milling



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HIGHLIGHTS

- Two step pretreatments had synergistic effects in increasing sugar yields.
- Hot water pretreated sample were directly milled without a separation step.
- Dilute acid pretreated sample was washed before milling due to low pH.
- Two step pretreatments reduced particle size and milling energy consumption.
- Acid with milling had similar or higher sugar yields than hot water with milling.

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ABSTRACT

Efficient pretreatment is essential for economic conversion of lignocellulosic feedstocks into monosaccharides for biofuel production. To realize high sugar yields with low inhibitor concentrations, hot water or dilute acid pretreatment followed by disk milling is proposed. Corn stover at 20% solids was pretreated with hot water at 160–200 °C for 4–8 min with and without subsequent milling. Hot water pretreatment and disk milling acted synergistically to improve glucose and xylose yields by 89% and 134%, respectively, compared to hot water pretreatment alone. Hot water pretreated (180 °C for 4 min) and milled samples had the highest glucose and xylose yields among all hot water pretreated and milled samples, which were comparable to samples pretreated with 0.55% dilute acid at 160 °C for 4 min. However, samples pretreated with 1% dilute acid at 150 °C for 4 min and disk milled had the highest observed glucose (87.3%) and xylose yields (83.4%).

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1. Introduction

In cellulosic bioethanol production processes, pretreatment disrupts the biomass cell walls and is necessary to improve accessibility of carbohydrases to cellulose and hemicellulose.

Abbreviations: AFEX, ammonia fiber explosion; ALS, acid insoluble lignin; ASL, acid soluble lignin; cSF, combined severity factor; DA, dilute acid; FPU, filter paper unit; GOS, glucooligosaccharide; HW, hot water; HMF, 5-hydroxymethylfurfural; HPLC, high performance liquid chromatography; LAP, laboratory analytical procedure; LSD, Fisher's protected least significant difference; NREL, National Renewable Energy Laboratory; WDM, wet disk milling; XOS, xylooligosaccharide.

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Pretreatment is the most critical processing step, influencing downstream processes such as conditioning of pretreated samples, recovery of coproducts, and wastewater treatment (Kumar and Murthy, 2011; Yang and Wyman, 2008). Pretreatments consist of either biological, chemical, and/or physical processes (Menon and Rao, 2012; Taherzadeh and Karimi, 2008).

Among various pretreatment methods, dilute acid pretreatment has been most studied because it is highly effective and directly end-hydrolyzes the hemicelluloses fraction (Yang and Wyman, 2008). However, dilute acid has several disadvantages. It often leads to the production of side products that inhibit enzymatic hydrolysis and fermentation and mitigating their effect often requires extensive conditioning of the hydrolysate. This increases sugar losses (Kumar and Murthy, 2011; Yang and Wyman, 2008). Also, dilute acid pretreatment is an expensive process with high capital costs for acquiring corrosion resistant reactors and high operation costs for chemicals, neutralization and disposal (Yang

and Wyman, 2008). As an alternative, hot water pretreatment induces similar chemical reactions as dilute acid pretreatment because of the increased hydronium ion concentration at high temperatures and pressures, but is more environmentally friendly and entails lower operation and capital costs (Mosier, 2013). A large fraction of hemicellulose is hydrolyzed to liquid soluble oligosaccharides, while a small fraction is hydrolyzed to monomeric sugars, preventing the formation of furan inhibitors (Mosier et al., 2005). However, aqueous pretreatment requires 20–50 °C higher temperatures and 5–15 min longer processing times than dilute acid pretreatment for optimal performance (Mosier, 2013).

Recently, combining physicochemical or chemical pretreatment and physical pretreatment has gained attention in cellulosic ethanol processing (Chen et al., 2013, 2014; Dondi et al., 2015; Inoue et al., 2008; Kim et al., 2016; Lin et al., 2010; Zhu et al., 2010). Among the many physical pretreatment methods described, mechanical grinding (refining) has been used in the pulp and paper industry to increase paper strength by exposing more fiber-fiber contact on specific surface area (Nakagaito and Yano, 2004). Ball milling, extrusion, PFI milling, Szego milling and disk milling are all examples of mechanical grinding techniques for wet samples. Disk milling is advantageous compare to other wet milling techniques because of economy and scalability (Barakat et al., 2013; Hiden et al., 2009).

Combining physicochemical or chemical pretreatment with mechanical grinding has synergistic effects to reduce particle size, decrease cellulose crystallinity, and increase accessible specific surface (Huo et al., 2013). As a result, combining pretreatments improves sugar yields in enzymatic saccharification, shortens overall saccharification time (Chen et al., 2013), and lowers needed enzyme loading (Inoue et al., 2008). Dilute acid pretreatment followed by mechanical grinding increased glucose yields up to 20% and xylose yields up to 14% compared for nonground corn stover (Chen et al., 2013). Also, mechanical grinding following physicochemical or chemical pretreatment reduced energy consumption for lodgepole pine size reduction by as much as 95% because of structural changes during chemical pretreatment (Zhu et al., 2010).

To develop a simple integrated two step pretreatment process and reduce costs, physicochemically or chemically pretreated samples should be processed directly by mechanical grinding without neutralization, separation or adjustment of solids loading. There have been few reports that describe the direct application of disk milling following a chemical pretreatment. Although hot water pretreatment is mild, samples in these reports were washed (Ertas et al., 2014; Zakaria et al., 2015) or filtered (Inoue et al., 2008; Sasaki et al., 2015) before milling. In this study, hot water pretreated samples were subjected to disk milling without a washing or neutralization step using corn stover at 20% solids. Disk milling was performed after hot water or dilute acid pretreatment to reduce milling energy consumption. In previous reports, biomass structures swelled, loosened, and formed pores during hot water or dilute acid pretreatment, which increased susceptibility to shear forces from disk milling (Lee et al., 2010; Zhu et al., 2010). The effects of two step pretreatment, hot water or dilute acid pretreatment followed disk milling, on enzymatic hydrolysis, size reduction and energy consumption were investigated. In addition, results from the hot water and dilute acid pretreatments combined with disk milling were compared for sugar yields. This is the first published study that compares hot water and dilute acid pretreated disk milled corn stover.

2. Materials and methods

2.1. Feedstock

Corn stover harvested in 2012 was obtained from the University of Illinois South Farm in Urbana, IL. Samples were oven dried at

49 °C until the moisture content in the samples was less than 5%. Samples were ground by a hammer mill (W-8-H, Schutte-Buffalo Hammermill, Buffalo, NY) using 3/32 in (2.38 mm) sieve size. Ground samples were stored in sealed containers at 4 °C.

2.2. Compositional analysis of raw and pretreated corn stover

Composition analysis of raw corn stover was performed following NREL protocols (website: http://www.nrel.gov/biomass/analytical_procedures.html, accessed February 2015). Water and ethanol soluble extractives were removed from the samples via a Soxhlet method adapted from “Determination of extractives in biomass (NREL/TP-510-42619)” to prevent interference with further analyses. Compositions of extractives free samples were analyzed by a two step acid hydrolysis procedure as described in “Determination of structural carbohydrates and lignin in biomass (NREL/TP-510-42618).” Briefly, the sample was mixed with 72% sulfuric acid for 2 h at 30 °C. The acid concentration was diluted to 4% with deionized water and heated at 121 °C for 1 h in an autoclave. After the two step hydrolysis, the sample was filtered by vacuum using filter crucibles. Filtered solids were analyzed for acid insoluble lignin (AISL) and ash contents by drying at 105 °C for 24 h followed by drying at 575 °C for 4 h. Filtrate was analyzed for acid soluble lignin (ASL) by recording absorbance at 205 nm and for sugar concentrations by HPLC (Aminex HPX-87P, Bio-Rad, Hercules, CA).

Composition analyses of pretreated samples were performed after separating pretreated liquid and solid by centrifugation. Monomeric sugars and inhibitor concentrations in the pretreatment liquor were analyzed directly by HPLC (Aminex HPX-87H, Bio-Rad, Hercules, CA). To measure oligomeric sugars in the pretreatment liquor, liquor was adjusted to a final concentration of 4% acid by adding 72% sulfuric acid. Prepared supernatant samples were autoclaved at 121 °C for 1 h as described in the NREL protocol, “Determination of sugars, byproducts and degradation products in liquid fraction process samples (NREL/TP-510-42623).” Samples were neutralized with barium carbonate to pH 5–6 and total sugar concentrations were measured by HPLC (Aminex HPX-87P). To obtain oligomeric sugar concentrations, monomeric sugar concentrations were subtracted from the measured total sugar concentrations.

Water insoluble solids were prepared by washing solids with 25 ml DI water, vortexing 60 s, and centrifuging at 3500 rpm (2000 × g) for 3 min, which was adapted from the NREL protocol, “Determination of insoluble solids of pretreated biomass material (NREL/TP-510-42627).” The washing step was repeated until the pH of the samples reached 5–7. Compositions of water insoluble solids were determined by the two step acid hydrolysis method as described in the raw corn stover composition analysis.

2.3. Hot water, dilute acid pretreatment and wet disk milling

Corn stover was pretreated in a stainless steel Parr reactor (20 l) (Model 4557, Parr Instruments, Moline, IL) fitted with an anchor stirrer. Stirrer speed was adjusted by a geared, direct drive motor (700 Series, Boston Gear, Charlotte, NC). The reactor was covered with a flexible mantle heater used as a heat source. A thermocouple was sealed in a 1/8” diameter stainless steel sheath and connected to a reactor controller (4848M, Parr Instruments, Moline, IL) which controlled temperature, stirring speed and monitor pressure.

All pretreatments were conducted at 20% solids content by mixing 400 g dry biomass with 1.6 l tap water or dilute acid. Five hot water pretreatment conditions were chosen at temperatures of 160–200 °C and operation times of 4–8 min. Detailed pretreatment conditions for hot water are summarized in Table 1. Dilute acid pretreatment conditions were modified from previous reports,

stating that high sugar yields were achieved with a combined severity factor (cSF) of 1.5 (Lloyd and Wyman, 2005; Schell et al., 2003). Here, cSF values of 1.46 and 1.01 were chosen as high and mild dilute acid severities to compare to hot water pretreatment alone. Corn stover was pretreated at 150 °C for 4 min with 1.0% w/w sulfuric acid (DA, 1.0% at 150 °C for 4 min) for cSF 1.46 and at 160 °C for 4 min with 0.55% w/w sulfuric acid (DA, 0.55% at 160 °C for 4 min) for cSF 1.01. Pretreated sample pH was measured and heating and cooling temperatures were recorded every 5 min. Pretreatments were performed one time at a given condition due to the large amount of samples used.

Disk milling (Quaker City grinding mill model 4E, Straub Co., Philadelphia, PA) was carried out after hot water or dilute acid pretreatment. To reduce water usage, disk milling was performed with the same solids loading (20%) as pretreatment. Hot water pretreated samples were milled without any washing or neutralization steps. Dilute acid pretreated samples were washed by vacuum filtration with 10 ml DI water per g of wet sample and dewatered to 20% solids content for milling. Wet samples (150 g) were fed into the mill. The clearance between stationary and rotating disks was zero (minimal gap). The output speed of the mill was 89 rpm. Samples were ground 9 times consecutively. Disk milling electrical energy usage was recorded by an electricity usage monitor (Kill A Watt® P4480, P3 International, New York, NY).

2.4. Hydrolysis

Following hot water or dilute acid pretreatment and disk milling, enzyme hydrolysis was conducted similar to the procedure discussed in “Low Solids Enzymatic Saccharification of Lignocellulosic Biomass (NREL/TP-5100-63351)” with modifications. Hydrolysis was conducted at 10% solids content in 50 ml centrifuge tubes (Corning Inc., NY). Samples with known moisture content, which were determined by “Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples NREL/TP-510-42621,” were added to preweighed tubes. Citrate buffer (pH 4.2, 1 M) was added to obtain a final concentration of 0.05 M. Per gram of dry biomass, 0.3 ml of cellulase (received from DSM Bio-based Products & Services (Elgin, IL, U.S.A.)) was added, which is a whole broth cocktail of thermophilic enzymes (52 g protein/L) of fungal origin.

Deionized water was added to bring the volume of the reaction to 10% solids content. Substrate blank flasks were prepared in a similar manner except without enzyme addition. Enzyme blank flasks were prepared consisting of all reaction constituents except substrate.

Hydrolysis was performed on all prepared samples with substrate blank and enzyme blank flasks in a water bath (Gyromax 939XL, Amerex Instruments, Inc., Lafayette, CA) set at 62 °C and 75 rpm. Aliquot samples (1 ml) were taken at 0 and 72 h for glucose and xylose determinations. Each sample was centrifuged at 10000 rpm (9300 × g) (Model 5415 D, Brinkmann-Eppendorf, Hamburg, Germany) and the supernatant was analyzed using HPLC. The glucose concentration in the enzyme blank flasks was subtracted from the glucose concentrations in the hydrolysis samples to remove background glucose concentration.

Enzyme hydrolyses were conducted in duplicates. For hot water pretreated samples, sugar yield was calculated as the ratio of the amount of monomer sugars present in hydrolysate and the corresponding carbohydrate concentrations in original samples (Agbor et al., 2011). Since dilute acid pretreated samples were washed after the pretreatment, sugar yields were calculated by combining sugar yields in soluble solids from pretreatment and insoluble solids from enzymatic hydrolysis.

Table 1
Hot water and dilute acid pretreatment conditions.

Sample	Temperature °C	Holding time min ^a	Dilute acid added % w/w	LogR''	LogR'	log R
HW at 160 °C for 4 min	160	4	NA ^b	5.61	-1.39	3.29
HW at 160 °C for 8 min	160	8	NA	5.82	-1.18	3.34
HW at 180 °C for 4 min	180	4	NA	6.74	-0.26	3.84
HW at 180 °C for 8 min	180	8	NA	7.24	0.27	4.09
HW at 200 °C for 4 min	200	4	NA	8.01	1.01	4.68
DA, 0.55% at 160 °C for 4 min	160	4	0.55	8.01	1.01	3.39
DA, 1.0% at 150 °C for 4 min	150	4	1.0	8.46	1.46	2.99

^a Holding time at reaction temperature.

^b Not applicable.

2.5. Analytical methods for sugars and inhibitors analysis

The prepared samples for carbohydrate and inhibitor analysis were filtered through a 0.2 µm syringe filter into 200 µL HPLC vials. Filtered liquid was injected into ion exclusion column HPX-87H or HPX-87P. The HPX-87H column used 5 mM sulfuric acid as eluent and operated at 50 °C, while the HPX-87P column used water as eluent and operated at 85 °C. Carbohydrates were measured with a refractive index detector (model 2414, Waters Corporation, Milford, MA). HPLC detection limits of sugar and ethanol were 0.001% w/w.

2.6. Particle size analysis

Samples were diluted with DI water to 1 mg/ml for size analysis. A particle size distribution range from 0.01 to 3500 µm was measured using a laser diffraction size analyzer (Malvern Mastersizer 3000 with Hydro EV unit, Malvern, UK) with 100 measuring points. Refractive index, absorption index and sample density were set to 1.40, 2.8 and 3 lb/ft³, respectively. Percent volume was acquired and used to determine the average particle size.

2.7. Severity factor

Generally, severity factor for hot water pretreatments is defined as (Overend et al., 1987):

$$R_0 = t \cdot \exp\{(T - T_R)/14.75\},$$

where t is reaction time (min), T is target temperature (°C) and T_R is reference temperature (100 °C). For dilute acid pretreatment, severity factor incorporates an acidity function and is named as combined severity factor (Chum et al., 1990):

$$\log R' = \log R_0 - pH,$$

where pH is that of the pretreated liquor. However, to compare enzyme digestibility from various catalytic pretreatments, severity factor can be calculated as (Pedersen and Meyer, 2010):

$$\log R'' = \log R_0 + |pH - 7|,$$

where pH is that of the pretreated liquor. Therefore, $\log R''$ was used in this study. In addition, in our case, reaction time was the sum of time at target temperature and corrected heating and cooling time whose rate constants were adjusted relative to the rate constant of actual time at target temperature.

$$\text{Corrected heating and cooling time} = \sum_0^n ((t_n - t_{n-1}) \cdot (k_n/k)),$$

where k_n is the rate constant for heating and cooling at time n and k is the rate constant at target temperature.

2.8. Statistical analysis

Reported results are means of duplicates and are on a dry weight basis. Fisher's protected least significant difference (LSD) was performed with 95% confidence level for pretreated samples with the same composition. Pearson's product moment correlation coefficient (r) was calculated, and significances were tested by a two tailed test with alpha of 0.05.

3. Results and discussion

3.1. Compositions of raw and hot water pretreated corn stover

Corn stover was comprised of 35.1% w/w glucan, 20.6% xylan and 14.5% lignin based on dry weight. The compositions were in agreement with previously reported values: 32–36% glucan, 16–22% xylan and 11–16% lignin (Chen et al., 2013; Weiss et al., 2010). The compositions of the insoluble solids recovered after hot water pretreatments are listed in Table 2, where they are ordered from lowest to highest severities. As the severity factor increased, glucan and lignin contents in the insoluble solids of the pretreated samples increased from 41.9 to 52.6% and from 21.2 to 37.9%, respectively. Glucan and lignin contents increased with increases in severity because of greater solubilization of hemicellulose during pretreatment. The hemicellulose (xylan) content of the insoluble solids decreased from 21.7% to 0% as severity factor increased from 5.61 (HW at 160 °C for 4 min) to 8.01 (HW at 200 °C for 4 min). Similar results were observed by Ertas et al. (2014) in regard to solubilization of hemicellulose.

Compositions in hot water pretreatment liquor corresponded with compositions in the hot water pretreatment insoluble solids (Table 3). As severity factor increased from 5.61 to 8.01, more acetic acid was produced, increasing from 1.00 to 6.88% w/w. Acetic acid arises from release of acetyl groups bonded to hemicelluloses and lowers the pH of the reaction. As a result, xylose (monomeric) in the pretreatment liquor increased from 0.64 to 2.83% w/w as severity factor increased from 5.61 (HW at 160 °C for 4 min) to 7.24 (HW at 180 °C for 8 min). However, at the highest severity factor tested for hot water pretreatment (HW at 200 °C for 4 min), xylose in the pretreatment liquor decreased. A similar trend was seen for xylooligosaccharide (XOS) in the pretreatment liquor, where the concentration increased until a severity factor of 6.74 (HW at 180 °C for 4 min) was achieved and thereafter declined. Xylose and XOS concentrations decreased because xylose degraded to furfural by an acid catalyzed reaction. At the harsher pretreatment conditions, furfural can be further converted into formic acid (Jonsson et al., 2013). Similar to xylose degradation, glucose degrades to 5-hydroxymethylfurfural (HMF) that can be converted into formic acid or levulinic acid at harsher pretreatment conditions. Samples pretreated at 200 °C for 4 min generated 15 fold higher formic acid concentrations compared to samples pretreated at 160 °C for 4 min. Also, 0.86% w/w HMF was detected in samples pretreated at 200 °C for 4 min.

3.2. Effects of disk milling on sugar yields

In the preliminary study, milling cycles of 3, 6 and 9 were evaluated using dilute acid pretreated samples. Each milling cycle means one pass through the mill. The sample with 6 milling cycles

Table 2

Compositions (% w/w) of insoluble solids from hot water or dilute acid pretreated samples.^a

Sample ^{b,c}	Glucan % w/w	Xylan % w/w	Lignin ^d % w/w
HW at 160 °C for 4 min	41.9 ± 0.16	21.7 ± 0.42	21.2 ± 0.02
HW at 160 °C for 8 min	42.3 ± 0.26	21.7 ± 0.33	22.0 ± 0.06
HW at 180 °C for 4 min	50.4 ± 0.98	12.3 ± 0.23	25.8 ± 0.17
HW at 180 °C for 8 min	52.6 ± 2.28	5.95 ± 0.23	31.8 ± 0.14
HW at 200 °C for 4 min	50.3 ± 1.98	BDL ^e	37.9 ± 0.12
DA, 0.55% at 160 °C for 4 min	51.9 ± 0.40	9.39 ± 0.40	27.8 ± 0.33
DA, 1.0% at 150 °C for 4 min	54.7 ± 0.67	7.71 ± 0.17	28.3 ± 0.38
LSD ^f ($\alpha = 0.05$)	3.01	0.606	0.653

^a Mean ± standard deviation.

^b Sample order is from the lowest to the highest severity factor.

^c HW indicates hot water and DA indicates dilute acid.

^d Lignin is sum of acid insoluble and acid soluble lignin.

^e Below HPLC detection limit (0.001% w/w).

^f Fisher's protected least significant difference.

had 30% higher glucose yields than the sample with 3 milling cycles. Glucose yields increased by an additional 10% after 9 milling cycles compared to 6 milling cycles. Therefore, 9 milling cycles were chosen in this study.

The total sugar yields after 72 h enzymatic hydrolysis of all samples are shown in Fig. 1, which were calculated based on original sample compositions. Compared to raw samples, glucose yields improved by 2.5 fold and xylose yields improved by 4.6 fold after wet disk milling (WDM). WDM treated corn stover samples had similar glucose yields (48.7%) as HW (180 °C for 4 min) treated sample (50.2%), which was the maximum glucose yield among samples treated with hot water alone. Disk milling itself is an effective pretreatment method to increase sugar yields, as previously reported (Hideno et al., 2009; Zhang et al., 2013). Mechanical grinding leads to Class I and II size reductions (Leu and Zhu, 2013). In class I size reduction, shear forces increase fiber external surface area via fiber separation, cutting, fragmentation and external fibrillation (delamination). In Class II size reduction, cell walls are deconstructed by breaking microfibril cross links and by compression induced internal fibrillation. Disk milling, ball milling, extrusion and PFI milling all induce Class II size reduction (Leu and Zhu, 2013). As a consequence of Class II size reduction, disk milled samples had comparable sugar yields to hot water pretreated samples (Fig. 1).

Hot water pretreatment followed by disk milling had synergistic effects in increasing sugar yields in enzymatic hydrolysis (Fig. 1). Across all hot water pretreatment conditions, disk milling increased glucose yields by 34–89% and xylose yields by 4–134%. Glucose and xylose yields of hot water pretreated samples without milling were from 26.5 to 50.2% and from 1.9 to 52.2%, respectively. With disk milling as post treatment, glucose yields ranged from 47.7 to 79.1%, while xylose yields ranged from 4.5 to 54.3%. HW (180 °C for 4 min) and WDM treated samples achieved the highest glucose and xylose yields.

Among hot water and disk milling treated samples, hot water pretreatment conditions above severity factor (LogR^v) of 6.74 had minimal improvement in xylose yield. In those samples, high concentrations of oligosaccharides were observed after hydrolysis. Assuming all oligosaccharides could be hydrolyzed to monomers, HW (180 °C for 4 min) and WDM treated would achieve 7.3 percentage points higher glucose yields (86.4%) and 18.1 percentage points higher xylose yields (72.4%). A large amount of oligosaccharides after hydrolysis might result from low hemicellulase activities. Qing and Wyman (2011) identified that XOS are strong inhibitors of cellulase activity. Increasing xylanase and β -xylosidase activities reduced inhibition by XOS and achieved high xylose monomer yields. A similar result was observed from

Table 3
Sugar and inhibitor concentrations (% w/w) in the hot water or dilute acid pretreatment liquor.^a

Sample ^{b,c}	Glucose % w/w	GOS ^d % w/w	Xylose % w/w	XOS ^d % w/w	Acetic acid % w/w	HMF ^e % w/w	Furfural % w/w	Formic acid % w/w
HW at 160 °C for 4 min	0.59 ± 0.04	2.90 ± 0.04	0.64 ± 0.04	2.65 ± 0.04	1.00 ± 0.08	BDL ^f	BDL	0.28 ± 0.02
HW at 160 °C for 8 min	0.61 ± 0.01	2.74 ± 0.03	0.68 ± 0.01	3.14 ± 0.05	0.96 ± 0.02	BDL	BDL	0.26 ± 0.02
HW at 180 °C for 4 min	0.32 ± 0.00	2.70 ± 0.00	1.25 ± 0.02	9.33 ± 0.04	1.71 ± 0.03	BDL	BDL	0.69 ± 0.01
HW at 180 °C for 8 min	0.34 ± 0.00	1.75 ± 0.07	2.83 ± 0.00	3.33 ± 0.18	2.49 ± 0.01	BDL	0.89 ± 0.02	1.16 ± 0.01
HW at 200 °C for 4 min	0.59 ± 0.02	0.70 ± 0.06	0.36 ± 0.00	0.26 ± 0.00	6.88 ± 0.02	0.86 ± 0.01	1.93 ± 0.01	4.09 ± 0.29
DA, 0.55% at 160 °C for 4 min	0.79 ± 0.10	1.89 ± 0.23	6.2 ± 0.84	5.18 ± 0.11	0.90 ± 0.12	BDL	1.05 ± 0.13	0.15 ± 0.02
DA, 1.0% at 150 °C for 4 min	2.32 ± 0.10	0.94 ± 0.06	14.9 ± 0.45	0.16 ± 0.22	1.68 ± 0.04	BDL	1.18 ± 0.02	0.16 ± 0.00
LSD ^g ($\alpha = 0.05$)	0.14	0.23	0.85	0.28	0.14	0.01	0.12	0.26

^a Mean ± standard deviation.

^b Sample order is from the lowest to the highest severity factor.

^c HW indicates hot water and DA indicates dilute acid.

^d Oligomeric sugars present in soluble solids of liquid pretreated samples. GOS indicates glucooligosaccharides and XOS indicates xylooligosaccharides.

^e 5-Hydroxymethylfurfural.

^g Fisher's protected least significant difference.

^f Below HPLC detection limit (0.001% w/w).

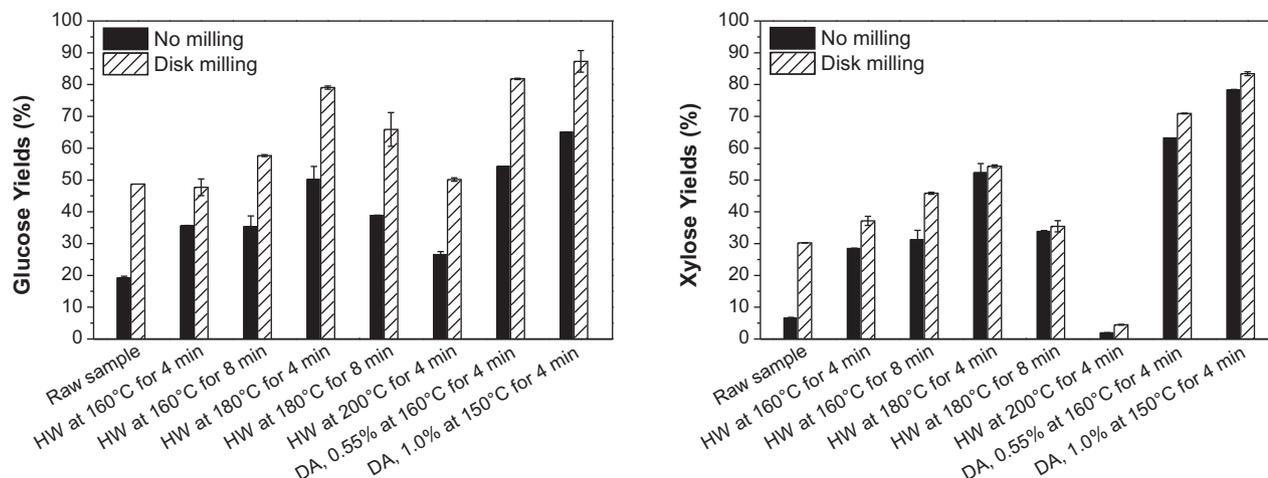


Fig. 1. Overall glucose and xylose yields after two step pretreatment and 72 h enzymatic hydrolysis. HW: hot water pretreatment, DA: dilute acid pretreatment. Sample order from left to right is from the lowest to the highest severity factor for the hot water pretreated samples. Yields are expressed as % of beginning carbohydrate extracted as monosaccharide. Standard deviations are included in graphs.

Kont et al. (2013) that oligosaccharides were poor substrates to cellulase and hemicellulase, but optimization of enzyme mixture could reduce production of oligosaccharides.

3.3. Effects of particle size and inhibitors on enzymatic hydrolysis

Hot water pretreatments reduced particle size (Fig. 2, blue traces). The average particle size of raw biomass was 735 μm (Table 4). The mean particle size decreased by 34–58% after hot water pretreatment. One narrow peak was observed in raw biomass; after hot water pretreatments, peaks were shifted to the left and bimodal distributions were observed. The smallest average particle size was found in hot water pretreated at 180 °C for 4 min (306 μm).

Disk milling reduced particle size (Fig. 2, red traces). The large particle peak in the bimodal distribution disappeared, and curves were left shifted further than those of nonmilled samples (Table 4). WDM treated samples (128 μm) had similar average particle size to HW (160 °C for 4 min) with WDM treated samples (105 μm), and HW (160 °C for 8 min) with WDM treated samples (130 μm). After disk milling, raw samples, HW (160 °C for 4 min) treated samples and HW (160 °C for 8 min) treated samples had average particle sizes of 128, 105, and 130 μm , respectively. Greater size reduction was achieved in samples hot water pretreated above

180 °C followed by disk milling, which had particle size below 60 μm . One possible explanation was that lignin was solubilized above 180 °C and formed droplets on the cell wall (Donohoe et al., 2008), which made pretreated biomass more susceptible to shear forces from disk milling.

Particle size reduction by milling did not explain completely the increased sugar yields. Average particle size and sugar yields showed negative correlation ($r = -0.43$). The smallest average particle size (36.6 μm) was achieved from HW (200 °C for 4 min) with WDM treated samples, but low glucose yields were obtained. Size reduction was an important parameter to increase enzyme digestibility, but crystallinity, surface area and pore volume also affected hydrolysis rate (Leu and Zhu, 2013). One possibility is that cell walls collapsed at the higher temperature, which limited enzyme access to the cellulose fibers. Moreover, components such as xylan and lignin are competitive inhibitors of cellulase, lowering their overall activity (Nakagame et al., 2011). Cellulase has higher adsorption affinity for xylan than glucan, and β -glucosidase has greater affinity for lignin than cellobiose (Qing and Wyman, 2011). In addition, byproducts from pretreatment, including phenolic compounds, organic acids, furan aldehydes, inorganic ions and bioalcohols, may act as competitive inhibitors to enzymes (Jing et al., 2009; Jonsson et al., 2013). Inhibitors generated during pretreatment affected sugar yields (Fig. 1 and Table 3), which were

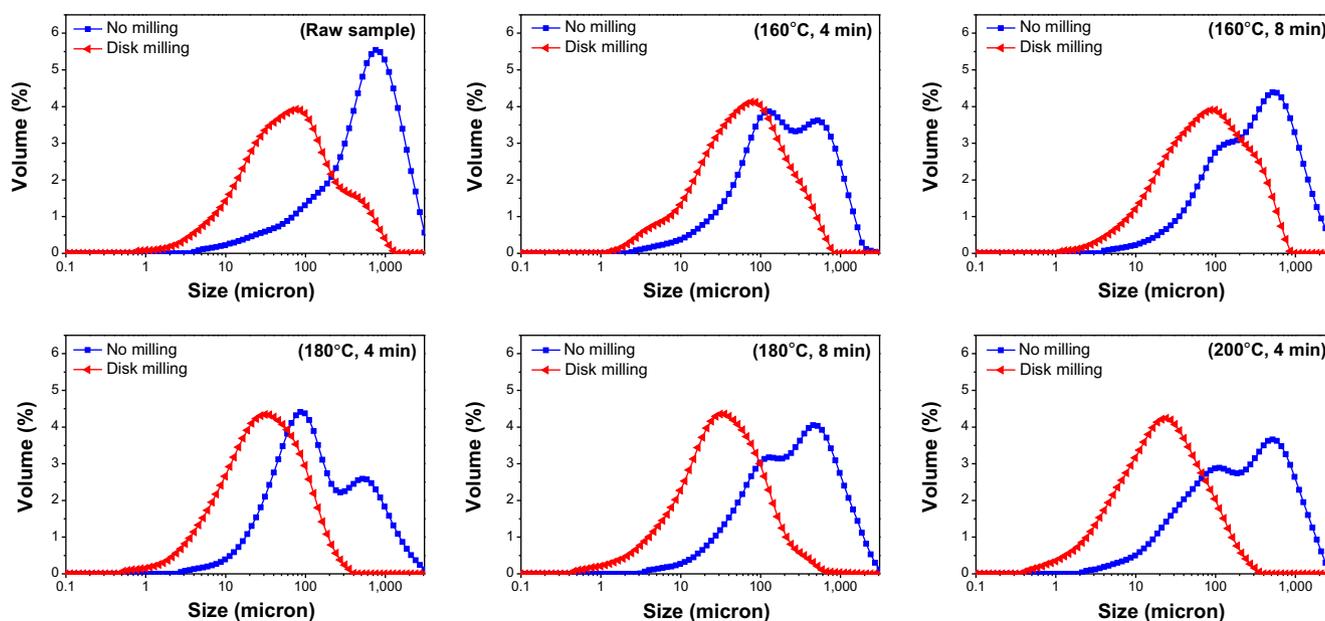


Fig. 2. Size reduction with and without disk milling after hot water pretreatment.

Table 4

Average particle size of hot water pretreated samples and milling energy consumption.

Sample ^a	Particle size before milling μm	Particle size after milling μm	Milling energy consumption ^c kWh/kg
Raw sample	735	128	16.3
HW ^b at 160 °C for 4 min	338	105	9.84
HW at 160 °C for 8 min	487	130	13.0
HW at 180 °C for 4 min	306	46.5	8.83
HW at 180 °C for 8 min	432	58.2	6.00
HW at 200 °C for 4 min	383	36.6	8.19

^a Sample order is from the lowest to the highest severity factor.

^b HW indicates hot water.

^c Total energy consumption to mill 9 cycles.

the same trends in sugar yields from hot water pretreated samples both with and without disk milling. Samples treated with HW at 180 °C for 4 min released the largest amount of oligomeric sugars (glucooligosaccharide (GOS) of 2.70% and XOS of 9.33%) (Table 3). As a result, fewer inhibitors were formed, and the highest glucose and xylose yields were achieved after hydrolysis. Even though HW at 180 °C for 8 min was the most effective condition to deconstruct biomass structure and resulted in high amount xylose release, relatively high amounts of inhibitors were generated, which negatively impacted sugar yields. In particular, sugar yields were decreased from samples in which furfural was detected (HW at 180 °C for 8 min and HW at 200 °C for 4 min).

3.4. Effects of hot water pretreatment on disk milling energy consumption

Advantages of disk milling after physicochemical pretreatment are reduction in energy consumption and increased sugar yield. Energy consumption of disk milling depends on process cycles,

disk gap, sample moisture and viscosity, initial particle size and type of feedstock (Chen et al., 2014; Hiden et al., 2009; Zhu et al., 2010). In this study, after 9 cycles of wet disk milling with minimal disk gap, raw corn stover with 20% moisture consumed 16.3 kWh per kg dry biomass, which is 25–172% higher energy consumption than hot water pretreated corn stover samples (Table 4). Physicochemical pretreatment before disk milling swelled the biomass structure and made it more susceptible to shear forces from disk milling (Zhu et al., 2010). Especially, hemicellulose removal during physicochemical pretreatment increased the pore size within the biomass and weakened the biomass matrix, which might reduce energy consumption for milling (Zhu et al., 2010). Energy consumption of HW at 160 °C for 4 min, HW at 160 °C for 8 min, HW at 180 °C for 4 min, HW at 180 °C for 8 min and HW at 200 °C for 4 min were 9.84, 13.0, 8.83, 6.00 and 8.19 kWh/kg dry corn stover, respectively (Table 4). Therefore, the high energy consumption required for disk milling of raw samples was reduced (63%) by sample pretreatment prior to disk milling. Energy consumption did not decrease as severity factor increased. This could be because sample viscosity changed by different pretreatment conditions. Similar observation was seen in a previous report that high viscose hot water pretreated samples had higher milling energy than low viscose superheated steam pretreated samples (Zakaria et al., 2015).

3.5. Comparison of hot water with wet disk milling treated samples and dilute acid with wet disk milling treated samples

Similar or higher glucan and lower xylan contents were observed in insoluble solids of dilute acid pretreated corn stover than hot water pretreated samples (Table 2). However, dilute acid pretreatment hydrolyzed more carbohydrates to monomeric sugars than hot water pretreatment (Table 3). The harsh DA (1.0% at 150 °C for 4 min) produced 2.32% w/w glucose and 14.9% w/w xylose, which were 7 and 12 fold higher, respectively, than sugars released from samples treated with HW at 180 °C for 4 min (Table 3). Similar to the effects of disk milling after hot water pretreatment on sugar yields, disk milling after dilute acid pretreatment improved glucose yield by 51% for DA (0.55% at 160 °C for 4 min) and 34% for DA (1.0% at 150 °C for 4 min) (Fig. 1). Mild DA

(0.55% at 160 °C for 4 min) with WDM treated samples had comparable glucose yields to HW (180 °C for 4 min) with WDM treated samples, which were 81.8 and 79.1%, respectively. The harsh dilute acid pretreatment condition (DA, 1.0% at 150 °C for 4 min) without disk milling, which produced one of highest sugar yields in previous reports (Lloyd and Wyman, 2005; Schell et al., 2003), achieved lower sugar yields than HW at 180 °C for 4 min with disk milling. Hot water pretreatment with disk milling was a good alternative process to dilute acid pretreatment alone. However, higher glucose yields (87.3%) and xylose yields (83.4%) were attained from harsh DA (1.0% at 150 °C for 4 min) with WDM treated samples compared to HW with WDM treated samples. This might be related to xylose and XOS concentrations in soluble solids of dilute acid pretreated samples (Table 3). Compared to HW at 180 °C for 4 min, harsh dilute acid pretreatment hydrolyzed xylan to mostly xylose and produced less XOS. Low XOS concentrations after dilute acid pretreatment might not affect enzymes activities extensively and resulted in high sugar yields.

Even though dilute acid with disk milling achieved higher glucose and xylose yields after enzymatic hydrolysis than hot water pretreatment with disk milling, hot water pretreatment did not require a special corrosion resistant reactor, and no washing or neutralizing step was needed before disk milling. The combined process of hot water pretreatment with disk milling is straightforward, and does not require a washing or neutralization step. The combined process of dilute acid pretreatment with disk milling requires a corrosion resistant reactor and a washing or neutralization step, but results in higher sugar yields than the hot water combined process.

4. Conclusion

Hot water or dilute acid pretreatment with disk milling had synergistic effects in increasing sugar yields. Sample with hot water and milling improved glucose yields by 89% and xylose yields by 134%. Particle size reduction after milling was not a primary factor affecting enzymatic digestibility; rather, inhibitors (acetic acid, furfural, HMF and formic acid) produced during pretreatment primarily affected sugar yields. Hot water pretreated milled samples and mild dilute acid pretreated milled samples achieved comparable glucose yields, which were lower than harsh dilute acid pretreated milled samples. Both combined processes showed promise toward ethanol production by increasing enzyme digestibility.

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References

Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B., 2011. Biomass pretreatment: fundamentals toward application. *Biotechnol. Adv.* 29, 675–685.

Barakat, A., de Vries, H., Rouau, X., 2013. Dry fractionation process as an important step in current and future lignocellulose biorefineries: a review. *Bioresour. Technol.* 134, 362–373.

Chen, X., Kuhn, E., Wang, W., Park, S., Flanagan, K., Trass, O., Tenlep, L., Tao, L., Tucker, M., 2013. Comparison of different mechanical refining technologies on the enzymatic digestibility of low severity acid pretreated corn stover. *Bioresour. Technol.* 147, 401–408.

Chen, X., Shekiri, J., Pschorn, T., Sabourin, M., Tao, L., Elander, R., Park, S., Jennings, E., Nelson, R., Trass, O., Flanagan, K., Wang, W., Himmel, M., Johnson, D., Tucker, M., 2014. A highly efficient dilute alkali deacetylation and mechanical (disc)

refining process for the conversion of renewable biomass to lower cost sugars. *Biotechnol. Biofuels* 7, 98.

Chum, H., Johnson, D., Black, S., Overend, R., 1990. Pretreatment-catalyst effects and the combined severity parameter. *Appl. Biochem. Biotechnol.* 24, 1–14.

Dondi, D., Zeffiro, A., Nola, P., Facchini, A., Arcioni, P., Malagodi, M., Licchelli, M., Nielsen, E., Buttafava, A., 2015. Structural modification of alfalfa stems during hot water and enzymatic hydrolysis for sugar production. *Cellulose* 22, 1853–1860.

Donohoe, B.S., Decker, S.R., Tucker, M.P., Himmel, M.E., Vinzant, T.B., 2008. Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment. *Biotechnol. Bioeng.* 101, 913–925.

Ertas, M., Han, Q., Jameel, H., Chang, H.-M., 2014. Enzymatic hydrolysis of autohydrolyzed wheat straw followed by refining to produce fermentable sugars. *Bioresour. Technol.* 152, 259–266.

Hideno, A., Inoue, H., Tsukahara, K., Fujimoto, S., Minowa, T., Inoue, S., Endo, T., Sawayama, S., 2009. Wet disk milling pretreatment without sulfuric acid for enzymatic hydrolysis of rice straw. *Bioresour. Technol.* 100, 2706–2711.

Huo, D., Fang, G., Yang, Q., Han, S., Deng, Y., Shen, K., Lin, Y., 2013. Enhancement of eucalypt chips' enzymolysis efficiency by a combination method of alkali impregnation and refining pretreatment. *Bioresour. Technol.* 150, 73–78.

Inoue, H., Yano, S., Endo, T., Sakaki, T., Sawayama, S., 2008. Combining hot-compressed water and ball milling pretreatments to improve the efficiency of the enzymatic hydrolysis of eucalyptus. *Biotechnol. Biofuels* 1, 2.

Jing, X., Zhang, X., Bao, J., 2009. Inhibition performance of lignocellulose degradation products on industrial cellulase enzymes during cellulose hydrolysis. *Appl. Biochem. Biotechnol.* 159, 696–707.

Jonsson, L., Alriksson, B., Nilvebrant, N.-O., 2013. Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnol. Biofuels* 6, 16.

Kim, S.M., Dien, B.S., Singh, V., 2016. Promise of combined hydrothermal/chemical and mechanical refining for pretreatment of woody and herbaceous biomass. *Biotechnol. Biofuels* 9, 1–15.

Kont, R., Kurašin, M., Teugias, H., Väljamäe, P., 2013. Strong cellulase inhibitors from the hydrothermal pretreatment of wheat straw. *Biotechnol. Biofuels* 6, 1.

Kumar, D., Murthy, G.S., 2011. Impact of pretreatment and downstream processing technologies on economics and energy in cellulosic ethanol production. *Biotechnol. Biofuels* 4, 27.

Lee, S.-H., Chang, F., Inoue, S., Endo, T., 2010. Increase in enzyme accessibility by generation of nanopore in cell wall supramolecular structure. *Bioresour. Technol.* 101, 7218–7223.

Leu, S.-Y., Zhu, J.Y., 2013. Substrate-related factors affecting enzymatic saccharification of lignocelluloses: our recent understanding. *Bioenergy Res.* 6, 405–415.

Lin, Z., Huang, H., Zhang, H., Zhang, L., Yan, L., Chen, J., 2010. Ball milling pretreatment of corn stover for enhancing the efficiency of enzymatic hydrolysis. *Appl. Biochem. Biotechnol.* 162, 1872–1880.

Lloyd, T.A., Wyman, C.E., 2005. Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. *Bioresour. Technol.* 96, 1967–1977.

Menon, V., Rao, M., 2012. Trends in bioconversion of lignocellulose: biofuels, platform chemicals & biorefinery concept. *Prog. Energy Combust. Sci.* 38, 522–550.

Mosier, N.S., 2013. Fundamentals of aqueous pretreatment of biomass. *Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals*. John Wiley & Sons Ltd, pp. 129–143.

Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., Ladisch, M.R., 2005. Optimization of pH controlled liquid hot water pretreatment of corn stover. *Bioresour. Technol.* 96, 1986–1993.

Nakagaito, A.N., Yano, H., 2004. The effect of morphological changes from pulp fiber towards nano-scale fibrillated cellulose on the mechanical properties of high-strength plant fiber based composites. *Appl. Phys. A* 78, 547–552.

Nakagame, S., Chandra, R.P., Kadla, J.F., Saddler, J.N., 2011. Enhancing the enzymatic hydrolysis of lignocellulosic biomass by increasing the carboxylic acid content of the associated lignin. *Biotechnol. Bioeng.* 108, 538–548.

Overend, R.P., Chornet, E., Gascoigne, J.A., 1987. Fractionation of lignocelluloses by steam-aqueous pretreatments [and discussion]. *Phil. Trans. R. Soc. Lond. A* 321, 523–536.

Pedersen, M., Meyer, A.S., 2010. Lignocellulose pretreatment severity – relating pH to biomatrix opening. In: *Papers from Symbiosis – The 14th European Congress on Biotechnology (Part 1)*, Barcelona, September, vol. 27, pp. 739–750.

Qing, Q., Wyman, C., 2011. Supplementation with xylanase and beta-xylosidase to reduce xylo-oligomer and xylan inhibition of enzymatic hydrolysis of cellulose and pretreated corn stover. *Biotechnol. Biofuels* 4, 18.

Sasaki, K., Tsuge, Y., Sasaki, D., Teramura, H., Inokuma, K., Hasunuma, T., Ogino, C., Kondo, A., 2015. Mechanical milling and membrane separation for increased ethanol production during simultaneous saccharification and co-fermentation of rice straw by xylose-fermenting *Saccharomyces cerevisiae*. *Bioresour. Technol.* 185, 263–268.

Schell, D.J., Farmer, J., Newman, M., McMillan, J.D., 2003. Dilute-sulfuric acid pretreatment of corn stover in pilot-scale reactor: investigation of yields, kinetics, and enzymatic digestibilities of solids. *Appl. Biochem. Biotechnol.* 105–108, 69–85.

Taherzadeh, M.J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. *Int. J. Mol. Sci.* 9, 1621–1651.

- Weiss, N.D., Farmer, J.D., Schell, D.J., 2010. Impact of corn stover composition on hemicellulose conversion during dilute acid pretreatment and enzymatic cellulose digestibility of the pretreated solids. *Bioresour. Technol.* 101, 674–678.
- Yang, B., Wyman, C.E., 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuel. Bioprod. Bioref.* 2, 26–40.
- Zakaria, M.R., Norrrahim, M.N.F., Hirata, S., Hassan, M.A., 2015. Hydrothermal and wet disk milling pretreatment for high conversion of biosugars from oil palm mesocarp fiber. *Bioresour. Technol.* 181, 263–269.
- Zhang, J., Wang, M., Gao, M., Fang, X., Yano, S., Qin, S., Xia, R., 2013. Efficient acetone–butanol–ethanol production from corncob with a new pretreatment technology–wet disk milling. *Bioenergy Res.* 6, 35–43.
- Zhu, W., Zhu, J.Y., Gleisner, R., Pan, X.J., 2010. On energy consumption for size-reduction and yields from subsequent enzymatic saccharification of pretreated lodgepole pine. *Bioresour. Technol.* 101, 2782–2792.