academicJournals

Vol. 14(39), pp. 2800-2811, 30 September, 2015
DOI: 10.5897/AJB2015.14806
Article Number: C5DDFF455642
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Fermentable sugars and microbial inhibitors formation from two-stage pretreatment of corn stalk with variation in particle size and severity factor

Nawa Raj Baral[1,2], Jianzheng Li[1]* and Ajay Kumar Jha[1]

[1]State Key Laboratory of Urban Water Resource and Environment, School of Municipal and Environmental Engineering, Harbin Institute of Technology, 73 Huanghe Road, Harbin 150090, P.R. China.
[2]Institute of Engineering, Tribhuvan University, Kathmandu, Nepal.

Received 18 June, 2015; Accepted 23 September, 2015

Microbial inhibitors including weak acids, furan derivatives and phenolic compounds are key problems of cellulosic bio-fuels production by fermentation. Most of these inhibitors are sugars and lignin degradation compounds, which are almost unavoidable during pretreatment processes. While, most of the one stage pretreatment has been conducted at high severity factors of 3.5 or more to get high sugar yield, with increase in severity factor, high concentration of microbial inhibitors were formed and significantly affected downstream biofuel yield. Thus, a two-stage pretreatment of corn stalk, hydrothermal followed by oxalic acid, under low severity factor and its enzymatic degradability was investigated in this study to identify fermentable sugar production and corresponding microbial inhibitors formation. Additionally, effect of equivalent severity factors of 2 to 3.5 and particle sizes of 1 to 35 mm were also studied systematically. Particle size of 15 mm was found as an optimum size at an equivalent severity factor of 2.5. Sugars 61.99 ± 0.03 g and inhibitors 5.12 ± 0.01 g from 100 g of corn stalk were obtained at the optimum particle size and pretreatment condition. The highest glucan conversion and recovery at the optimum conditions were 92.95± 0.08 and 78.42± 0.07%, respectively. Overall, the two-stage pretreatment process with the larger particle size and low equivalent severity factor could be an alternative to reduce microbial inhibitors formation and excessive biomass processing cost.

Key words: Bio-fuel, corn stalk, pretreatment, particle size, microbial inhibitors, fermentable sugars.

INTRODUCTION

Worldwide concern of biofuels including ethanol, butanol and biodiesel from cheap and abundant lignocellulosic biomass is increased rapidly because of price volatility, rising demand, environmental issues, energy security and sustainability of limited fossil fuel (Vancov et al., 2012; Liu et al., 2013; Saha et al., 2013). Lignocellulosic biomass, such as corn stalk, has shown a great potential to contribute alternative vehicular fuels demand due to its

*Corresponding author. E-mail: jianzhengli@hit.edu.cn. Tel: +86 451 86283761.

immediate availability and almost useless for other purposes (Qureshi et al., 2007; Chundawat et al., 2011; Cheng et al., 2012; Bondesson et al., 2013; Zhang et al., 2014). Corn stalk is mainly composed of cellulose, hemi-cellulose, and lignin, where cellulose fibers are surrounded by hemi-cellulose and lignin matrix. Lignin is the main barrier for bio-fuel production from corn stalk (Alonso et al., 2013) because the lignin cannot be directly consumed by fermenting microorganisms. Thus, pretreatment is an essential step for disrupting the complex lignin-cellulose-hemicellulose structure, which can enhance fermentable sugar production during the successive enzymatic hydrolysis process (Kumar et al., 2009).

Over the years, several pretreatment processes such as the physical (milling and grinding), the chemical (hydrothermal, high pressure steam explosion, acid, alkali, oxidizing agents, and organic solvents, ammonia fiber explosion), the biological (fungi, actinomycetes, and bacteria) as well as a combination of those pretreatment approaches have been investigated on variety of feedstocks (Taherzadeh and Karimi 2007; Zheng et al., 2009a; Kumar et al., 2009; Zheng et al., 2009b; Yang et al., 2013). Among them, chemical pretreatment process is popular, while most researchers did not emphasize physical pretreatment due to the high cost and low sugar yield and biological pretreatment due to time intensive. Specifically, dilute acid treatment is a common pretreatment process, which produces more sugar by disrupting the crystalline structure of cellulose, increasing porosity and making enzymatic hydrolysis easier (Vancov et al., 2012). In addition to acidic pretreatment, hydro-thermal pretreatment is also considered as an eco-friendly treatment process because the pretreatment medium contains feedstock and water only that avoids corrosion and acid sludge. The energy efficiency of these pretreatment processes primarily depends on pretreat-ment temperature and retention time. In general, one stage pretreatment processes require high pretreatment temperature and longer time to destroy lignin barrier and enhance successive enzymatic hydrolysis. However, a severe pretreatment condition, that is, high temperature and long duration, leads to formation of sugar and lignin degradation compounds. These sugar and lignin degradation products including weak acids, furan derivatives and phenolic compounds are toxic to most of the common bio-fuel producing bacteria, which are used during fermentation process (Tadesse and Luque, 2011; Guo et al., 2012; Um and van Walsum, 2012). For instance, inhibitors such as phenolic compounds, and furan derivatives like furfural and hydroxymethylfurfural (HMF) have remarkable impact on butanol production (Qureshi et al., 2008; Guo et al., 2012; Baral et al., 2014). Some inhibitor detoxification processes are available, which require additional cost. In fact, up to 13% sugars loss has been reported during the overliming process (Palmqvist and Hahn-Hägerdal, 2000; Guo et al., 2012; NREL, 2011), which is the most common detoxification method.

Thus, an effective pretreatment process is a necessity which should provide high sugar yield and low sugar as well as lignin degradation byproducts, that is, microbial inhibitors. The pretreatment process should also be inexpensive and less energy intensive (Qin et al., 2012; Liu et al., 2013). It is obvious that the larger particle size reduces extensive biomass preparation energy and equipment cost as well as the microbial inhibitors can be reduced under mild pretreatment conditions. However, single chemical pretreatment may not destroy lignin barrier of large particle size under the mild pretreatment condition. Thus, a two-stage pretreatment process, hydrothermal treatment followed by oxalic acid, was investigated in this study. The former, that is, hydrothermal pretreatment, was selected because of an eco-friendly process. And, oxalic acid is selected because it is known as a suitable pretreatment process that can effectively reduced the extensive sugar and lignin degradation compounds even under mild conditions (Lee et al., 2011; Qin et al., 2012). Additionally, xylans, which forms a barrier that restricts cellulases access to cellulose (Kreuter, 1996), can be effectively removed by using the mild oxalic acid pretreatment process (Lee et al., 2011; Mtui, 2012). The foremost objective of current study was to examine fermentable sugars and inhibitors from corn stalk with variation in particle size and pretreatment conditions. Additionally, best particle size among selected particle sizes was identified based on products and byproducts, which opens up future extensive research possibilities to optimize particle size and their mechanism study for high sugar yield and low microbial inhibitors formation.

MATERIALS AND METHODS

Corn stalk collection and preparation

Corn stalk with moisture content of 30 to 37% was collected from Xinfazhen village, Harbin, China (45.75°N, 126.63°E). The corn stalk was reduced into different sizes including 1 mm (Φ 1 mm), 5 mm (5 × 5 × 3.5 mm), 10 mm (10 × 5 × 3.5 mm), 15 mm (15 × 5 × 3.5 mm), 25 mm (25 × 5 × 3.5 mm) and 35 mm (35 × 5 × 3.5 mm). Each batch size was dried in oven at 45 ± 1°C until the moisture content reduced to 5 to 7%. Structural composition of each batch size was analyzed after further reduction into required size, around 1 to 2 mm (Hames et al., 2008). Each batch size was separately analyzed to reduce the error associated with sample size preparation. And then, these different batches were stored at 4 ± 1°C in separate sealed packets for further processes.

Two-stage pretreatment

Figure 1 illustrates a schematic of the two-stage pretreatment process adopted in this research, where liquid hydrolysate was extracted after each pretreatment stage to prevent formation of the degradation compounds. Solid loading rate of 10% (10 g dry corn stalk/100 ml of deionized water and/or oxalic acid) was used for

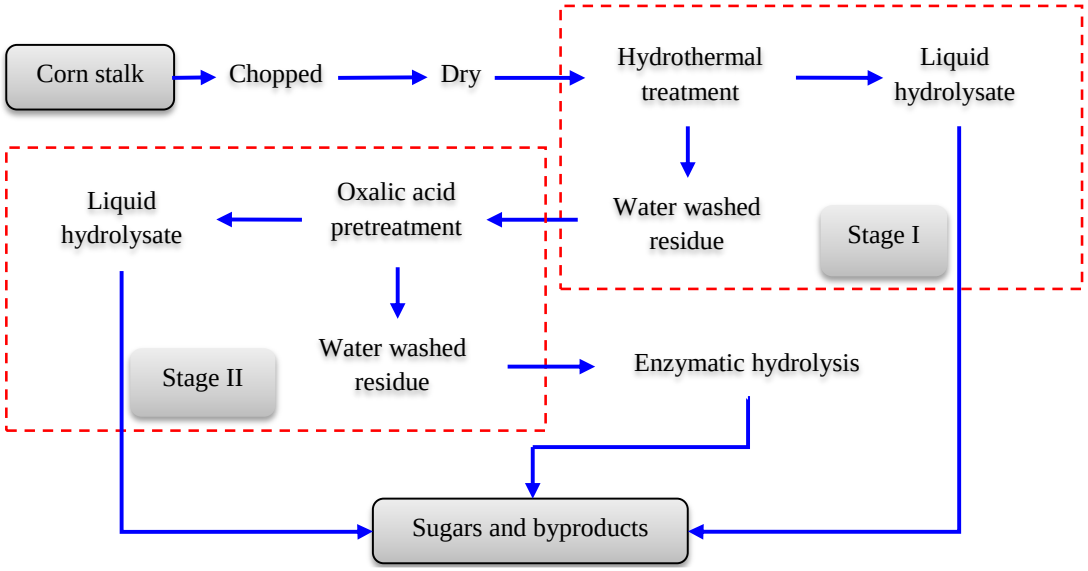

Figure 1. Schematic of a two-stage pretreatment of corn stalk.

both stages of pretreatment, that is hydrothermal and oxalic acid treatment for each of the selected particle size. The sample of each size was prepared in iodometric flask with 250 ml capacity. The pretreatment parameters including temperature, time and chemical concentration (if applicable) were finalized after thorough analysis of sugars yield and corresponding inhibitors formation reported in the previous studies (Liu et al., 2013; Saha et al., 2013; Palmqvist and Hahn-Hägerdal, 2000; Lee et al., 2011; Qin et al., 2012). The hydrothermal pretreatment was performed in an autoclave at 125 ± 1°C for 30 min at a pressure of about 0.23 MPa. The heating and cooling times of the flasks were not considered as a pretreatment time, even though it took about 23 min to reach the final temperature of 125 ± 1°C and about 45 min to cool to room temperature of 25 ± 1°C. After the hydrothermal pretreatment, liquid hydrolysate was separated using cotton fabric filter and sample was taken for analysis. The residual solid stalk was washed with tap water and squeezed using the same filter. Then, the solid was oven dried before oxalic acid pretreatment to maintain same solid loading rate of 10% (*w/v*). Oxalic acid concentration 0.3 mol/l and the same temperature and time as hydrothermal pretreatment were used for further structural breakdown of the pretreated corn stalk. After oxalic acid pretreatment, the liquid hydrolysate was taken for analysis. The solid residue was washed with water until neutral pH and oven dried at 105 ± 1°C, which was further used for enzymatic hydrolysis. Additionally, the structural composition of solid residue was analyzed after each pretreatment and enzymatic hydrolysis. All the samples and residue were stored at -20 ± 1°C for further uses.

Furthermore, experiments were conducted to observe effect of variation in retention time on particle size. The best particle size at a pretreatment condition of 125 ± 1°C and 30 min and two equal range particle sizes, one above and one below the best size, were considered. For these three selected particle sizes, apart from pretreatment time of 30 min, other pretreatment durations of 10, 92 and 290 min for each pretreatment stage were also investigated. All the other pretreatment conditions and methodology were identical with the first experiment.

Enzymatic hydrolysis

The enzymatic digestibility tests of the pretreated corn stalk were carried out using the National Renewable Energy Laboratory (NREL) standard protocol (LAP-009) (Hames et al., 2008). Cellulase used during enzymatic saccharification was purchased from Imperial Jade Biotechnology Co., Ltd., China. The average activity of the enzyme was 100 filter paper units per ml (FPU/ml). The cellulase was loaded at 4.5 FPU/g glucan (Lin et al., 2011) and hydrolysis was performed at 45 ± 1°C, 100 rpm (Saha et al., 2013) in a rotary shaker (FLY-2102C, Shanghai Shenxian Thermostatic Equipment Factory, China). All the tests were run in triplicate. Liquid samples were taken periodically for reducing sugar analysis. The samples were filtered by eight layers of gauze and centrifuged at 8000 rpm for 10 min (Lin et al., 2011).

Analytical methods

The moisture content, extractive and composition of raw as well as pretreated corn stalk was determined according to the laboratory analysis protocol (LAP) of the NREL (Hames et al., 2008). Sugars and microbial inhibitors such as acetic acid, formic acid, levulinic acid, furfural and HMF of after each stage pretreatment and enzymatic saccharification were determined using high performance liquid chromatography (HPLC), Shimadzu, Japan and following NREL protocol. Aminex HPX-87P and Aminex HPX 87H with refractive index detector (RID-10A) were used to determine sugars and inhibitors concentration, respectively. The operating conditions of HPLC were identical with NREL protocol and columns guidelines. Oligomeric sugars in the hydrolysate were determined according to LAP-014 of NREL (Hames et al., 2008). Additionally, UV Spectrophotometer (Shimadzu, Japan) was used to determine soluble lignin at a wavelength of 320 nm and following NREL protocol (Hames et al., 2008)..

Calculation

The combined effect of temperature (T) and residence time (t) for any pretreatment process can be expressed as severity factor (SF). The standard expression of SF is reported elsewhere (Lloyd and Wyman, 2005; Saha et al., 2013):

Table 1. Moisture content and extractives of corn stalk in the present research.

Sample size (mm)	Mass (g/100 g of raw corn stalk)	
	Moisture content	Extractives*
1	5.21 ± 0.09	15.54 ± 0.07
5	5.37 ± 0.07	15.17 ± 0.01
10	5.43 ± 0.01	13.62 ± 0.02
15	5.37 ± 0.01	12.39 ± 0.03
25	5.68 ± 0.03	13.02 ± 0.01
35	5.48 ± 0.01	12.99 ± 0.02

*Water (filtered with 0.2 µm), and ethyl alcohol (95% pure) were used to remove extractives.

$$SF = \log_{10}\left[\int_0^t exp\left\{\frac{T-100}{14.75}\right\}dt\right]$$

For a constant pretreatment temperature, the combined effect of temperature and time of a two-stage pretreatment process can be expressed as:

$$\text{Equivalent}SF = \log_{10}\left[exp\left\{\frac{T-100}{14.75}\right\}\left\{\int_0^{t_1} dt + \int_0^{t_2} dt\right\}\right]$$

In which T is the pretreatment temperature in °C, t_1 is the pretreatment time for first stage in min; that is, hydrothermal pretreatment time, and t_2 is the pretreatment time for second stage in min; that is, oxalic acid pretreatment time. The effect of variation in pretreatment time was compared based on the equivalent SF of the two-stage pretreatment process. Moreover, glucan and xylan recovery after the two-stage pretreatment process and conversion during enzymatic hydrolysis were determined. The equation for the recovery and conversion along with correction factors are available elsewhere (Qin et al., 2012). Finally, the total sugar was determined by addition of glucose, xylose and arabinose yield after each stage.

RESULTS AND DISCUSSION

Composition of corn stalk

Main stem of corn stover, corn stalk, was used in this study was dried for composition analysis and further treatment. The moisture content of each particle size is summarized in Table 1 along with the standard deviation. The results were within the range of NREL guidelines, that is, below 10%, for the composition analysis (Hames et al., 2008). The non-structural materials from biomass, also known as extractives, were removed prior to analysis to prevent interference with later analytical steps. Those were mainly water soluble and ethanol soluble materials. The extractives of all selected particle size batches are also presented in Table 1. The emphasis was given to make homogeneous mixture of collected corn stalks, even though there was about 13% variation within particle size batches. Such variation may be due to corn variety because they were collected from two different fields of Xinfazhen village, Harbin, China (45.75°N, 126.63°E) at the same season. Previous studies also found the extractives in a range of 11.2 to 24% based on raw corn stover (Qin et al., 2012; Liu et al., 2013). Thus, the data summarized in Table 1 are consistent with the previous studies. Table 2 illustrates an overview of corn stalk composition. The main desirable constituent is glucan followed by xylan, arabinan, mannan and galactan. Lignin, the third major fraction on weight basis after glucan and xylan, is undesirable fraction for bio-fuel production. Slight variation mainly in glucan, xylan and lignin was found in each size batch. It might be due to heterogeneous mixture of corn variety during batch size preparation; however, these data are almost similar to previous study (Donghai et al., 2006), where glucan, xylan, arabinan, mannan and galactan of corn stalk were reported to be 36.5, 21.6, 3.2, 2.4 and 1.7%, respectively. Additionally, lignin, ash and acetate content of corn stalk in this study were also consistent when compared to the previous studies (Donghai et al., 2006; Tamaki and Mazza, 2010; Lee et al., 2011). Furthermore, the composition of corn stalk is also comparable with corn stover (Bura et al., 2009; Aden and Foust, 2009). NREL (2011) evaluated the compositions of 508 commercial corn stover samples collected from 47 sites. They found 32 g glucan, 19 g xylan, 18 g extractives and 13 g lignin based on 100 g raw corn stover. Compared to their study, glucan, xylan, and lignin concentrations were found higher in this study. These variations might be due to corn variety, region, weather, soil type, and fertilization practices.

Composition of liquid hydrolysate

Hydrothermal treatment

Baral et al. (2014) reviewed pretreatment time and temperature severely impacts on inhibitors formation. In addition to these pretreatment conditions, particle size also significantly impact on biomass processing cost, that is, handling cost and milling cost. On the other hand, single stage pretreatment at lower temperature and shorter time might not be effective to destroy lignin barrier. Thus, wide range of particle sizes, 1 to 35 mm, and two-stage pretreatment at low SF were investigated to reduce processing cost and microbial inhibitor formation. Hydrothermal treatment was performed as a first phase of the two-stage pretreatment to decompose complex lignin barrier of corn stalk. This is generally known as a cheaper, noncorrosive and environment friendly treatment process. The sugar composition in the liquid hydrolysate after hydrothermal pretreatment with different particle sizes is summarized in Table 3, along with standard deviation of the three observations. The fermentable sugars on the hydrolysate components were varied with respect to the particle size. The lowest and

Table 2. Composition of corn stalk expressed as g/100 g of dry raw corn stalk.

Sample size (mm)	Glucan (g)	Xylan (g)	Galactan (g)	Arabinan (g)	Mannan (g)	Lignin* (g)	Ash (g)	Acetate (g)
1	37.39 ±0.04	22.88 ±0.04	0.95 ±0.01	2.44 ±0.02	1.16 ±0.02	15.69 ±0.06	4.05 ±0.02	2.07 ±0.01
5	35.87 ±0.02	22.47 ±0.01	0.88 ±0.00	2.34 ±0.01	1.10 ±0.02	15.41 ±0.03	4.33 ±0.02	2.04 ±0.01
10	34.71 ±0.02	22.32 ±0.07	0.93 ±0.00	2.32 ±0.03	1.14 ±0.02	18.01 ±0.07	4.45 ±0.02	2.06 ±0.02
15	36.16 ±0.06	22.38 ±0.02	0.91 ±0.01	2.15 ±0.03	1.13 ±0.02	17.05 ±0.09	4.44 ±0.01	2.03 ±0.01
25	35.32 ±0.03	23.08 ±0.02	0.92 ±0.03	2.33 ±0.01	1.13 ±0.01	17.39 ±0.04	4.49 ±0.02	2.06 ±0.06
35	35.34 ±0.07	25.08 ±0.09	0.73 ±0.01	3.17 ±0.02	0.98 ±0.02	16.72 ±0.03	4.50 ±0.01	2.08 ±0.02

*It consists of both acid soluble and insoluble lignin.

Table 3. Sugar composition in liquid fraction after hydrothermal pretreatment (g/100 g of dry raw corn stalk) at 125°C and 30 min.

Sample size (mm)	Glucose (g)	GO (g)	Xylose (g)	XO (g)	Arabinose (g)	AO (g)
1	2.28 ± 0.07	0.03 ± 0.09	4.56 ± 0.01	0.66 ± 0.02	0.52 ± 0.02	0.09 ± 0.07
5	2.72 ± 0.01	0.05 ± 0.01	5.17 ± 0.02	0.74 ± 0.02	0.42 ± 0.05	0.03 ± 0.06
10	2.44 ± 0.07	0.39 ± 0.07	3.80 ± 0.01	1.00 ± 0.01	0.54 ± 0.01	0.11 ± 0.01
15	1.77 ± 0.04	0.36 ± 0.01	4.04 ± 0.08	1.49 ± 0.03	0.08 ± 0.01	0.34 ± 0.06
25	1.54 ± 0.01	0.92 ± 0.01	2.45 ± 0.03	1.64 ± 0.02	0.30 ± 0.05	0.14 ± 0.03
35	1.67 ± 0.01	0.51 ± 0.01	2.34 ± 0.02	1.27 ± 0.04	0.28 ± 0.01	0.241 ± 0.02

GO = Glucose oligomers; XO = xylose oligomers; AO = arabinose oligomers.

Table 4. Sugar composition in liquid fraction after oxalic acid pretreatment (g/100 g of dry raw corn stalk) at 125°C and 30 min.

Sample size (mm)	Glucose (g)	GO (g)	Xylose (g)	XO (g)	Arabinose (g)	AO (g)
1	4.45 ± 0.01	0.92 ± 0.06	11.36 ± 0.05	1.82 ± 0.07	1.29 ± 0.02	0.02 ± 0.06
5	4.53 ± 0.01	0.01 ± 0.03	12.05 ± 0.07	1.63 ± 0.03	1.96 ± 0.06	0.02 ± 0.06
10	3.90 ± 0.08	1.11 ± 0.03	12.12 ± 0.03	1.00 ± 0.03	2.96 ± 0.02	0.04 ± 0.03
15	5.16 ± 0.04	1.20 ± 0.03	13.74 ± 0.01	1.00 ± 0.04	2.65 ± 0.01	0.05 ± 0.01
25	4.87 ± 0.06	0.16 ± 0.05	12.30 ± 0.08	0.70 ± 0.08	2.02 ± 0.01	0.03 ± 0.03
35	4.89 ± 0.06	0.18 ± 0.01	12.02 ± 0.02	1.09 ± 0.03	2.54 ± 0.02	0.03 ± 0.03

GO = Glucose oligomers; XO = xylose oligomers; AO = arabinose oligomers.

highest glucose productions were obtained at the particle sizes of 25 and 5 mm, respectively. Xylose was also found highest in the particle size of 5 mm, while the lowest was the 35 mm. Xylose oligomer was found more than glucose and arabinose oligomer and increased with the particle size except the particle size of 35 mm. Glucose, xylose and arabinose oligomer were also varied greatly with the particle size. These results suggest larger particle size is reluctant to hydrolysis at lower SF and smaller particle size (that is, 1 mm), and sugar might be degraded into microbial inhibitors, including HMF and furfural. High xylan degradation was observed during hydrothermal pretreatment when compared to glucose. It was hard to find a previous study on hydrothermal pretreatment with variation in particle sizes; however, the result was almost similar with steam explosion pretreatment of corn stover at 200 ± 1°C and 5 min (Liu et al., 2013).

Oxalic acid treatment

The hydrothermal pretreatment has been initially applied prior to acid hydrolysis of corn stalk. A major barrier of hydrothermal treatment is low sugar yield (Donghai et al., 2006). Therefore, a successive oxalic acid pretreatment was performed to enhance the sugar yield from the pretreated corn stalk. The results show that oxalic acid had a higher hydrolysis efficiency in comparison to sulfuric acid when applied under the same severity conditions (Lee et al., 2011). Table 4 demonstrates the monomeric and ologomeric sugar in liquid hydrolysate

Table 5. Total inhibitors formation after hydrothermal and oxalic acid treatment (g/100g of dry raw corn stalk) at 125ºC and 30 min.

Sample size (mm)	Weak acids			Furan derivatives		Soluble lignin (g)
	Acetic acid (g)	Formic acid (g)	Levulinic acid (g)	Furfural (g)	HMF (g)	
1	2.80 ± 0.01	0.00 ± 0.0	0.12 ± 0.0	0.67 ± 0.01	0.03 ± 0.0	0.72 ± 0.03
5	2.99 ± 0.05	0.00 ± 0.0	0.10 ± 0.01	0.58 ± 0.07	0.04 ± 0.0	0.51 ± 0.01
10	3.21 ± 0.04	0.29 ± 0.01	0.08 ± 0.0	0.41 ± 0.04	0.04 ± 0.0	0.43 ± 0.01
15	3.22 ± 0.09	0.33 ± 0.01	0.12 ± 0.01	0.62 ± 0.03	0.04 ± 0.0	0.79 ± 0.03
25	3.26 ± 0.09	0.00 ± 0.0	0.16 ± 0.01	0.45 ± 0.04	0.03 ± 0.0	0.62 ± 0.01
35	3.01 ± 0.03	0.28 ± 0.0	0.07 ± 0.0	0.41 ± 0.0	0.00 ± 0.0	0.51 ± 0.01

after oxalic acid treatment of the pretreated corn stalk, along with standard deviation of three observations. Xylose was a foremost constituent of the hydrolysate followed by glucose and arabinose. Oxalic acid has been reported as a better chemical for xylose yield (Qin et al., 2012). With small variation, glucose and xylose concentrations in the hydrolysate were increased with particle size up to 15 mm and then decreased. Data obtained in this research were comparable to the previous literatures in which different pretreatment conditions were used (Lee et al., 2011; Qin et al., 2012). Overall, xylan was removed when oxalic acid was added and this could enhance digestibility of cellulose during enzymatic hydrolysis.

Inhibitor formation after biphasic pretreatment

Pretreatment parameters play vital roles in avoiding degradation of hexose and pentose sugar as well as lignin into undesirable byproducts. Most of the byproducts are toxic to bio-fuel producing microbes. Weak acid furan derivatives and acid soluble lignin were determined in this research. Total of each inhibitor after hydrothermal and consecutive oxalic acid treatment are shown in Table 5. Unexpectedly, these inhibitors were higher at particle size 15 mm that also has higher sugar concentration. The similar situation was found in previous study (Liu et al., 2013). Low concentration of formic acid, levulinic acid and furfural were found during analysis. Those inhibitors were insignificant in some particle sizes (Table 5). Acetic acid was formed due to degradation of hemicelluloses and acetate, which was increased with particle size except particle size of 35 mm. There were no regular trends for HMF, furfural and acid soluble lignin. HMF and furfural concentrations were below 1 g/l and consistent with the previous study (Qin et al., 2012).

Enzymatic hydrolysis of pretreated corn stalk

Cellulase was used for enzymatic saccharification of pretreated corn stalk. One of the objectives of this study was to reduce enzyme loading rate. Low cellulase loading rate of 4.5 FPU/g glucan was tested to observe enzymatic degradability of pretreated corn stalk. In general, low enzyme loading rate requires longer retention time to hydrolyze the pretreated biomass. Previous study (Saha and Cotta, 2008) reported enzymatic hydrolysis at 45°C was better than 50°C for longer reaction time, thus, enzymatic hydrolysis was carried out at 45°C in the current study. The major solid residue after two-stage pretreatment was glucan. Figure 2 presents glucan conversion with particle sizes and hydrolysis time. When the pretreated corn stalk was hydrolyzed for 48 h with 4.5 FPU/g glucan, the glucan dissolution was approached to about 40% in the particle sizes of 15 and 35 mm.

During initial 24 h, glucan conversion was not dependent on particle size. Nearly 65% of the glucan from 15 mm particle size was converted to fermentable glucose in 120 h when compared to about 40% conversion from particle size of 1 mm at the same time. These results indicated that glucan conversion increased gradually with the time. It might be a result of low enzyme concentration. The hydrolysis was slowed down after 192 h that may be due to remaining highly crystalline part of corn stalk. The highest glucan conversion of 92.95% was found at the particle size of 15 mm. This conversion was better than the previous study of steam explosion corn stover at SF 3.64 (Liu et al., 2013). However, only 58.15% glucan conversion was observed at the particle size of 1 mm under the similar condition. Glucan conversion was remarkably improved in this study even at low enzyme loading rate in comparison to single pretreatment with any methods and similar SF based on review provided by Baral et al. (2014). This may be because of better disruption of crystalline structure after two-stage pretreatment. Previous study (Rollin et al., 2011) also reported lignin and lignin-like compounds were major obstacles of enzymatic degradability.

The results of enzymatic hydrolysis of pretreated corn stalk make known that higher hydrolysis efficiency can be achieved with larger particle sizes, which ultimately enhance pretreatment performance. Previous research has also reported improved enzymatic performance with

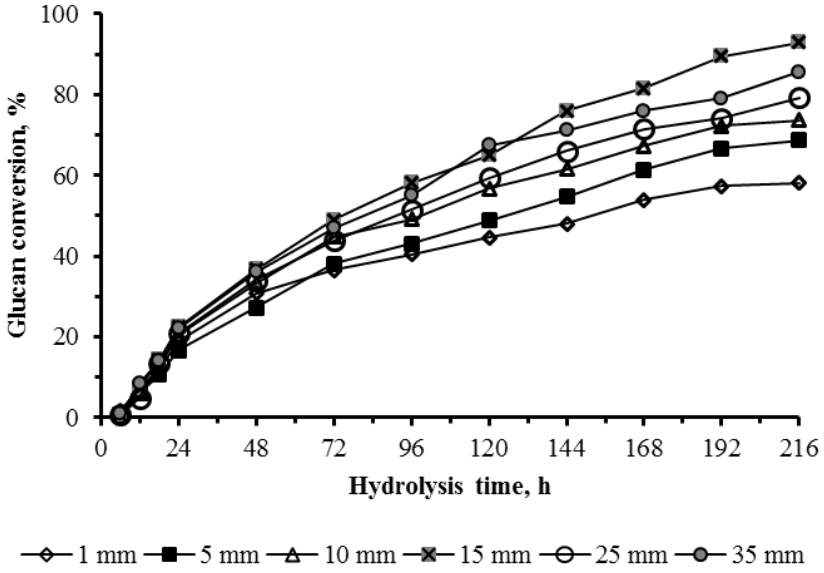

Figure 2. Glucan conversion of pretreated corn stalk at cellulose loading rate of 4.5 FPU/g glucan.

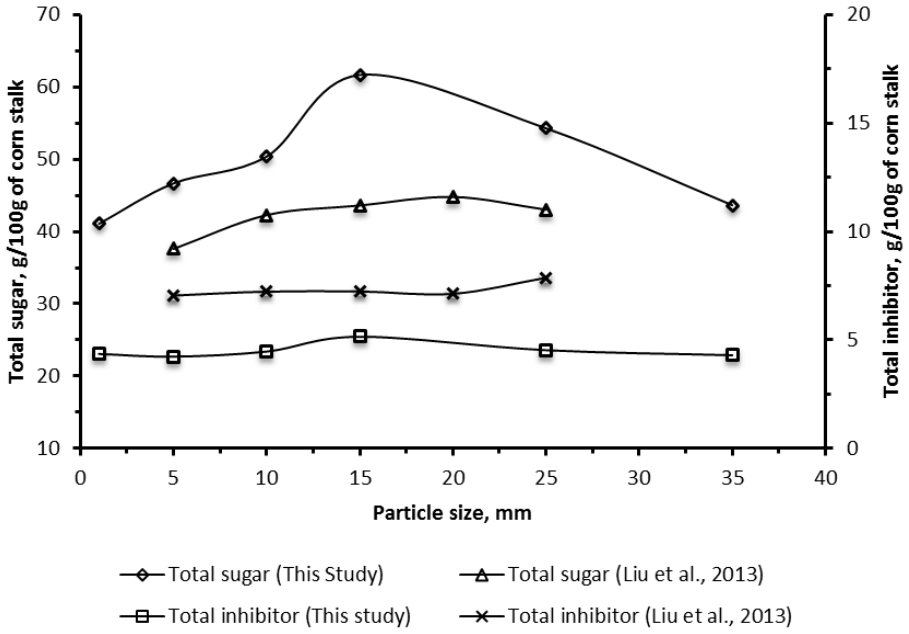

Figure 3. Total sugar and inhibitor after the two-stage pretreatment (at equivalent SF 2.5) and hydrolysis (at 4.5 FPU/g glucan for 216 h). The total sugar includes glucose, xylose and arabinose. Additionally, total inhibitor consists of acetic acid, formic acid, levulinic acid, furfural, HMF and soluble lignin. The present result is compared with previous study. Their results are after steam explosion pretreatment (at SF of 3.64) and enzymatic hydrolysis (60 FPU/g glucan for 168 h) of corn stover. They also considered similar sugar composition but did not include levulinic acid and soluble lignin in total inhibitor.

increase of biomass particle size (Liu et al., 2013). It is clear that efficiency of enzymatic hydrolysis is strongly influenced by surface area of particle. The overall performance including pretreatment and enzymatic hydrolysis in terms of total sugar and inhibitor is presented in Figure 3. The results obtained in this research were compared with the previous research of steam explosion pretreatment at 200°C and 5 min followed

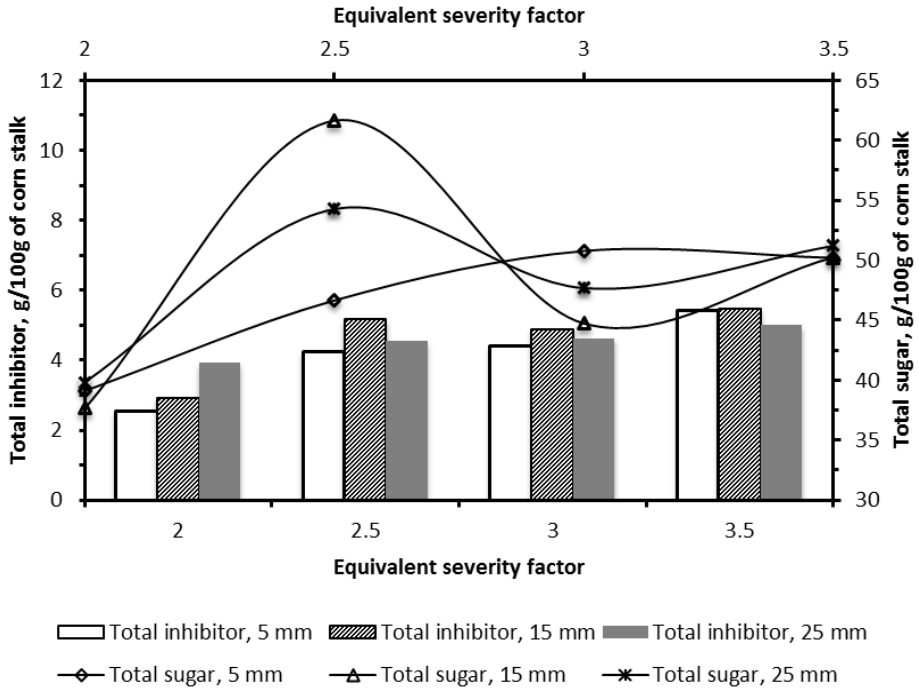

Figure 4. Effect of equivalent SF on sugar and inhibitor. The total sugar includes glucose, xylose and arabinose and total inhibitor includes acetic acid, formic acid, levulinic acid, furfural, HMF and soluble lignin.

by enzymatic hydrolysis (60 FPU/g corn stover) (Liu et al., 2013). In contrast to the previous study, this study showed that total sugar yield was increased whereas total inhibitor was decreased significantly; though the trends were almost similar (Figure 3). In the present study, total sugar yield showed an increasing trend with particle size up to 15 mm and then decreased. Total inhibitor yield was also followed the same trend. For 15 mm particle size total sugar and inhibitor yield were 61.99 and 5.12 from 100 g of corn stalk, respectively. A sugar yield as low as 41.06 g/100 g of corn stalk was observed at a particle size of 1 mm.

The total sugar in this research was also greater than the previous researches with hydrothermal and acidic pretreatment (Avci et al., 2013; Saha et al., 2013). The overall analysis of this research and comparison with previous studies suggests that two-stage pretreatment with larger particle size (about 15 mm) will be one of the promising alternatives in terms of pretreatment performance and commercial bio-fuel production. Larger particle sizes about 10 to 15 mm are energy efficient and save time before pretreatment when compared to the smaller ones.

Effect of severity factor on sugar and inhibitor yield

As discussed earlier, particle size of 15 mm was observed as an optimum size at an equivalent SF of 2.5. In order to investigate the effects of SF on sugar production and microbial inhibitor formation, two other equal range particle sizes of 5 and 25 mm were further investigated along with 15 mm size. The results are presented in the Figure 4. In the case of 5 mm particle size, total sugar gradually increased up to equivalent SF 3.0 and then almost constant with increase in SF. In contrast, total sugar for particle sizes 15 and 25 mm increased rapidly with increase in equivalent SF up to 2.5 and then decreased sharply until SF reached to 3.0. Finally, it became almost steady after the SF of 3.0. In addition to sugar production, total inhibitors increased with the increase in equivalent SF. Variation of total inhibitors within particle sizes at a particular equivalent SF were almost insignificant. These results suggest that lower SF is not sufficient to destroy lignin barrier, specifically for large particle size. Whereas at the higher SF more sugar degradation products were formed. Zhang et al. (2012) suggested that a smaller particle size was beneficial to enzymatic hydrolysis.

On the other hand, Liu et al. (2013) found that smaller particle sizes can cause high degradation of hemicelluloses and with increase in particle sizes the pretreatment process can destroy the lignin barrier of outer surface only. Delignification is beneficial for sugar production, which can be improved with decreasing particle size and increasing SF; however, high degradation of hemicelluloses and lignin cause formation of microbial inhibitors. Thus, proper SF and particle size is

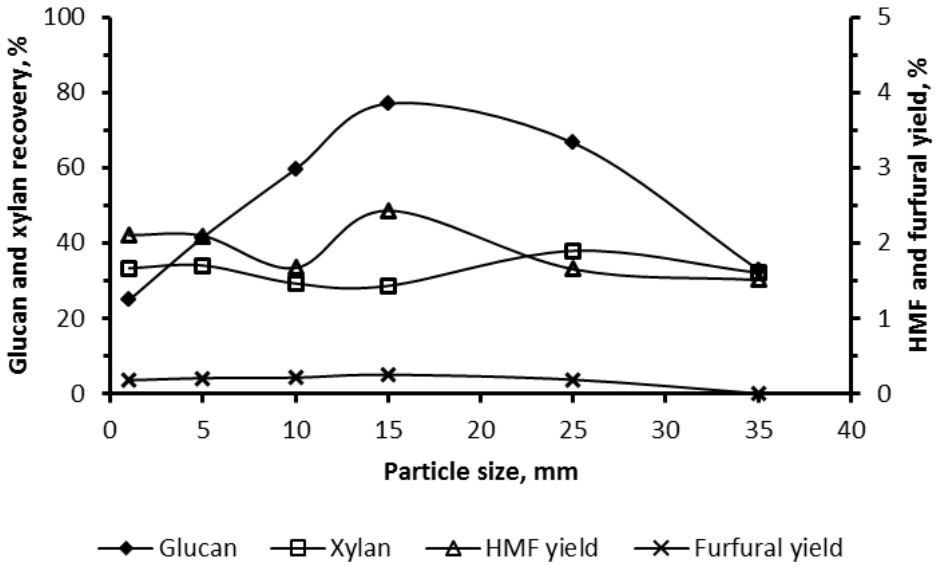

Figure 5. Effect of particle size on glucan and xylane recovery as well as HMF and furfural yield

essential for the effective enzymatic hydrolysis. This study also suggests that increase in surface area due to large particle size does not necessarily improve enzymatic hydrolysis. Two important facts were noted from this study. First, the peak of total sugar shifted towards the lower SF with increase in particle sizes. Secondly, at higher equivalent SF total sugar production was nearly equal and it was independent on particle size. In the single stage pretreatments, higher SF is essential to disrupt lignin barrier, which ultimately increase inhibitors formation and energy cost. The heating cost and sugar degradation products can be reduced using two-stage pretreatment with a low SF. It also helps to improve sugar concentration. However, proper economic analysis is necessary to justify the overall results.

Effect of particle size and severity factor on yield and recovery

Glucan and xylan recovery as well as HMF and furfural yield with particle size are presented in Figure 5. The glucan recovery followed a parabolic path with the maximum recovery of 78% for 15 mm particle size that was significantly higher when compared to other sizes. HMF and furfural yield were also more at 15 mm particle size. However, the differences were insignificant. Importantly, the xylan recovery of 15 mm particle size was lower when compared to other sizes. Loss of more xylan might increase porosity on the pretreated biomass and improve enzymatic hydrolysis. Thus, higher glucan and lower xylan recovery of 15 mm particle size might be one of the reasons of high sugar yield. Generally, performance of pretreatment and enzymatic hydrolysis was determined by glucose and xylose yield that ensure optimum utilization of raw biomass. Figure 6 illustrates glucose and xylose yields from the two-stage pretreatment followed by enzymatic hydrolysis processes. Glucose yield was increased with equivalent SF and became steady after SF 3.0. The trend was consistent with the previous study (Liu et al., 2013) and could be improved by increasing enzyme loading rate. Particle size of 15 mm had the highest glucose yield followed by 25 and 5 mm.

Maximum glucose yield of 90% was obtained at SF 2.5. It was found that glucose yields were more or less invariant with particle sizes at higher SF (>3). Xylose yield was also increased with particle size except 25 mm. It reached the highest yield at SF2.5 and then decreased slowly. This result was also consistent with previous study (Qin et al., 2012). However, rapid declination was observed after SF 2.5. It may be due to ineffective pretreatment and degradation of xylose into inhibitors. Optimum xylose yield of 94% was observed at a particle size and SF of 15 and 2.5 mm, respectively.

Overall mass balance

A schematic description of overall mass balance for particle size of 15 mm is shown in Figure 7. This description illustrates the pretreatment conditions and the amounts of major products and byproducts before and after the two-stage pretreatment as well as enzymatic hydrolysis. The mass balance was prepared based on the composition analysis of initial and recovered solid residue as well as liquid hydrolysis after each stage. The results showed that, when compared with the xylan, both

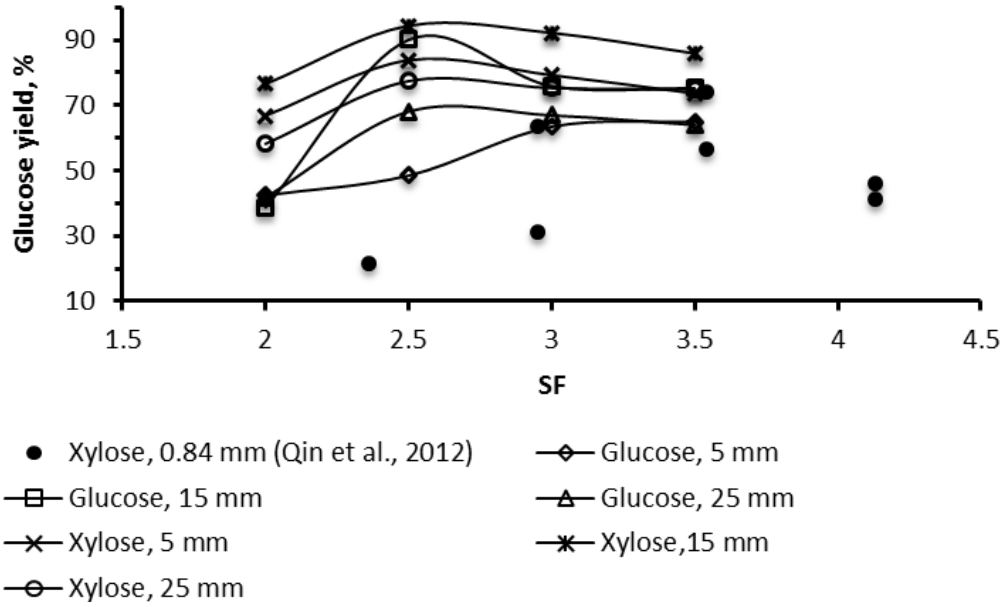

Figure 6. Glucose and xylose yield with variation in particle size and SF. Present study was based on equivalent SF of the two-stage pretreatment. Result of previous study were evaluated based on SF after oxalic acid pretreatment (130 to 190°C for 30 min with acidic concentration of 50 to 90 mM).

pretreatments preserve most of the glucan. Specifically, more xylan was lost during oxalic acid pretreatment. Approximately 96% glucan and 78% xylan were recovered during hydrothermal pretreatment. Additionally, recovery of glucan and xylan were decreased to 81.89 and 23.68%, respectively, during successive oxalic acid pretreatment. The higher loss in xylan during oxalic acid pretreatment has enhanced the enzymatic hydrolysis. Thus, only 4.5 FPU/g glucan can effectively hydrolyze the remaining solid residue during enzymatic hydrolysis process. About 85% of glucan was converted into glucose during enzymatic hydrolysis. About 92% lignin recovery was found during pretreatment process. It might be due to low SF of 2.21 during each pretreatment stage. Overall, the two-stage pretreatment and enzymatic hydrolysis produced 34.85 g of glucose, 23.98 g of xylose and 3.12 g of arabinose from 100 g oven dry (about 5% moisture content) corn stover. In addition to sugar, inhibitors including 3.22 g acetic acid, 0.33 g formic acid, 0.12 g levulinic acid, 0.44 g soluble lignin, 0.57 g furfural and 0.04 g HMF were also formed during the two-stage pretreatment process.

The total sugar (~62 wt%) found in this study was higher than average total sugar of the previous studies with hydrothermal pretreatment (~50 wt%) (Feng et al., 2014; Lloyd and Wyman, 2005; Mosier et al., 2005); and with oxalic acid pretreatment (~52 wt%) (Feng et al., 2014; Mtui, 2012; Qin et al., 2012). Additionally, total microbial inhibitors of ~5 g/l found in this study was lower than average total inhibitors of ~8 g/l reported in the previous single stage pretreatments (Feng et al., 2014; Qin et al., 2012). Thus, the two-stage pretreatment, hydrothermal followed by oxalic acid, collectively could destroy lignin barrier at low SF and with less degradation of sugars and lignin itself into microbial inhibitors. As a result, this pretreatment process could produce more fermentable sugars with low microbial inhibitors concentration. While, low SF used in this study could also save some cost of heating and cooling agents additional equipment and water requirement might be few challenges when compared to single stage pretreatment. Nevertheless, the two-stage pretreatment used in this study was advantageous in terms of sugar yield and microbial inhibitors formation when compared to similar single stage pretreatments.

Conclusion

Overall sugar production with two-stage pretreatment was dependent on both particle size and severity factor, whereas microbial inhibitors formation was specifically dependent on severity factor only. The total sugar of 41.07 to 61.99 g and inhibitor of 4.23 to 5.12 g were observed from 100 g raw corn stalk when particle sizes varied from 1 to 35 mm and at an equivalent severity factor of 2.5, while this pretreatment resulted more than two times xylose fraction in liquid hydrolysate when compared to glucose. Results show that choice of particle size and corresponding equivalent severity factor could be very crucial to obtain optimum sugar. The optimum performance of the two-stage pretreatment process was

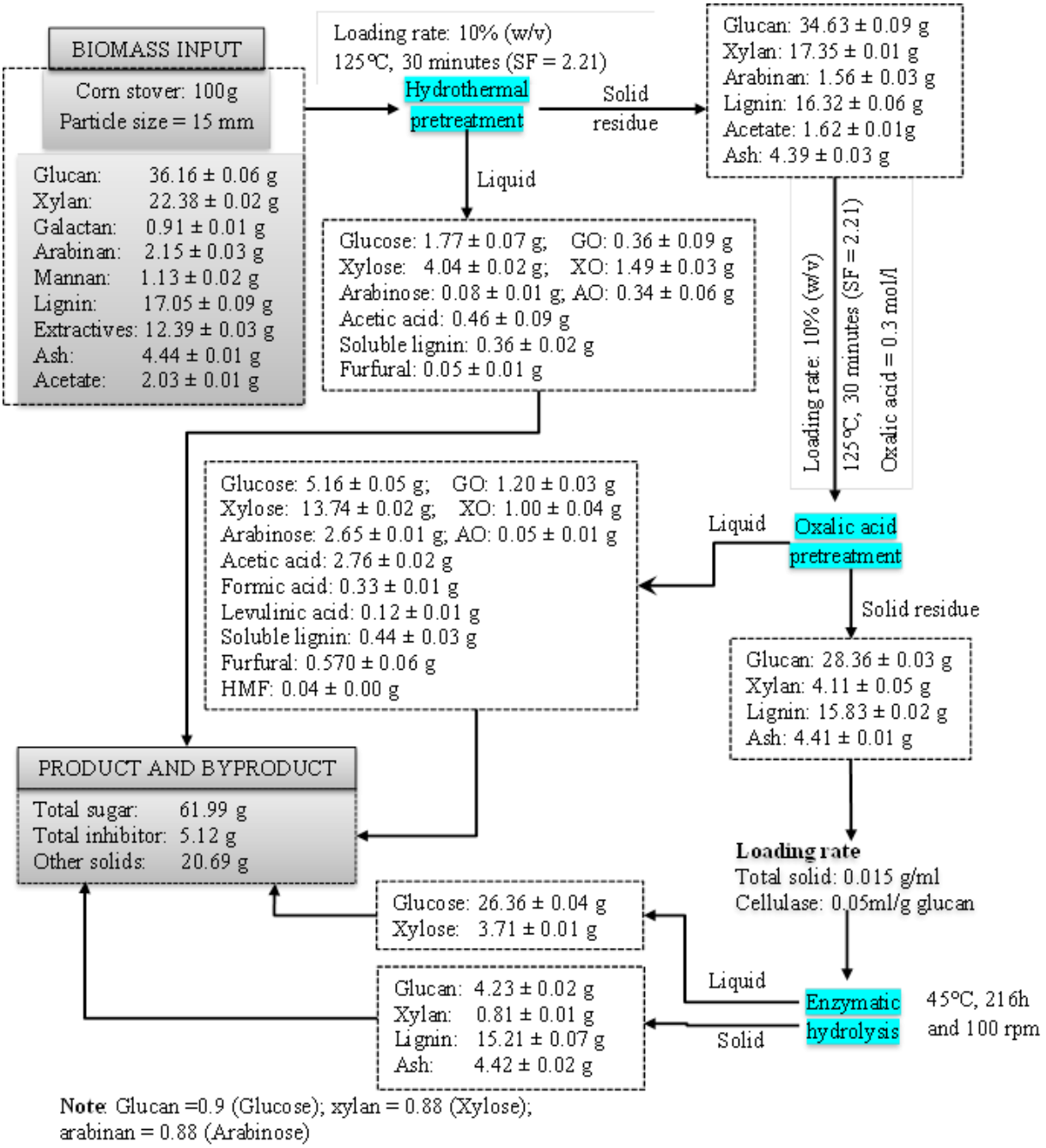

Figure 7. Mass balance during the two-stage pretreatment and enzymatic hydrolysis of corn stalk at particle size of 15 mm. The measured parameters are given in this mass balance. Mannose and galactose were not considered in the mass balance due to low concentration. Extractives were considered as a waste.

obtained at a particle size of 15 mm and an equivalent severity factor of 2.5. These results definitely assist to reduce excessive milling, detoxification cost and toxic effects of sugar and lignin degradation products on bio-fuels producing microbes. Thus, two-stage pretreatment with low severity factor and large particle size could be one of the possible options to optimize sugar production and reduce microbial inhibitor formation.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

This work was financially supported by the National

Natural Science Foundation of China (Grant No. 51478141), and State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology (Grant No. 2013DX11).

REFERENCES

Aden A, Foust T (2009). Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. Cellulose 16:535-545.

Alonso DM, Wettstein SG, Mellmer MA, Gurbuz EI, Dumesic JA (2013). Integrated conversion of hemicellulose and cellulose from lignocellulosic biomass. Energy Environ. Sci. 6:76.

Avci A, Saha BC, Kennedy GJ, Cotta MA (2013). Dilute sulfuric acid pretreatment of corn stover for enzymatic hydrolysis and efficient ethanol production by recombinant Escherichia coli FBR5 without detoxification. Bioresour. Technol. 142:312-319.

Baral NR, Li J, Jha AK (2014). Perspective and prospective of pretreatment of corn straw for butanol production. Appl. Biochem. Biotechnol. 172:840-853.

Bondesson PM, Galbe M, Zacchi G (2013). Ethanol and biogas production after steam pretreatment of corn stover with or without the addition of sulphuric acid. Biotechnol. Biofuels 6:11.

Bura R, Chandra R, Saddler J (2009). Influence of Xylan on the Enzymatic Hydrolysis of Steam-Pretreated Corn Stover and Hybrid Poplar. Biotechnol. Prog. 25(2):315-322, 315-322.

Cheng CL, Che PY, Chen BY, Lee WJ, Lin CY, Chang JS (2012). Biobutanol production from agricultural waste by an acclimated mixed bacterial microflora. Appl. Energy 100:3-9.

Chundawat SPS, Donohoe BS, da Costa Sousa L, Elder T, Agarwal UP, Lu F, Ralph J, Himmel ME, Balan V, Dale BE (2011). Multi-scale visualization and characterization of lignocellulosic plant cell wall deconstruction during thermochemical pretreatment. Energy Environ. Sci. 4:973.

Donghai SU, Junshe S, Ping L, Yanping L (2006). Effects of different pretreatment modes on the enzymatic digestibility of corn leaf and corn stalk. Chinese J. Chem. Eng. 14:796-801.

Feng L, Qin L, Liu Z, Dong C, Li B, Yuan Y (2014). Combined severity during pretreatment chemical and temperature on the saccharification of wheat straw using acids and alkalis of differing strength. BioResource 9:24-38

Guo T, Tang Y, Zhang QY, Du TF, Liang DF, Jiang M, Ouyang PK (2012). Clostridium beijerinckii mutant with high inhibitor tolerance obtained by low-energy ion implantation. J. Ind. Microbiol. Biotechnol. 39:401-407.

Hames B, Ruiz R, Scarlata C, Sluiter A, Sluiter J, Templeton D (2008). Preparation of Samples for Compositional Analysis Laboratory Analytical Procedure (LAP). http://www.nrel.gov/docs/gen/fy08/42620.pdf

Kreuter J (1996). Nanoparticles and microparticles for drug and vaccine delivery. J. Anat. 189 (Pt 3:503-505).

Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009). Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. Ind. Eng. Chem. Res. 48:3713-3729.

Lee JW, Houtman CJ, Kim HY, Choi IG, Jeffries TW (2011). Scale-up study of oxalic acid pretreatment of agricultural lignocellulosic biomass for the production of bioethanol. Bioresour. Technol. 102:7451-6.

Lin Y, Wang J, Wang X, Sun X (2011). Optimization of butanol production from corn straw hydrolysate by Clostridium acetobutylicum using response surface method. Chin. Sci. Bull. 56:1422-1428.

Liu ZH, Qin L, Pang F, Jin MJ, Li BZ, Kang Y, Dale BE, Yuan YJ (2013). Effects of biomass particle size on steam explosion pretreatment performance for improving the enzyme digestibility of corn stover. Ind. Crops Prod. 44:176-184.

Lloyd TA, Wyman CE (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.

Mosier N, Hendrickson R, Ho N, Sedlak M, Ladisch MR (2005). Optimization of pH controlled liquid hot water pretreatment of corn stover. Bioresour. Technol. 96:1986-1993.

Mtui GYS (2012). Oxalic acid pretreatment, fungal enzymatic saccharification and fermentation of maize residues to ethanol. Afr. J. Biotechnol. 11:843-851.

NREL (2011). Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol U.S. Department of Energy. http://www.nrel.gov/biomass/pdfs/51400.pdf

Palmqvist E, Hahn-Hägerdal B (2000). Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition. Bioresour. Technol. 74:25-33.

Qin L, Liu ZH, Li BZ, Dale BE, Yuan YJ (2012). Mass balance and transformation of corn stover by pretreatment with different dilute organic acids. Bioresour. Technol. 112:319-26.

Qureshi N, Saha BC, Cotta MA (2007). Butanol production from wheat straw hydrolysate using Clostridium beijerinckii. Bioprocess Biosyst. Eng. 30:419-427.

Qureshi N, Saha BC, Hector RE, Cotta MA (2008). Removal of fermentation inhibitors from alkaline peroxide pretreated and enzymatically hydrolyzed wheat straw: Production of butanol from hydrolysate using Clostridium beijerinckii in batch reactors. Biomass Bioenergy 32:1353-1358.

Rollin JA, Zhu Z, Sathitsuksanoh N, Zhang YHP (2011). Increasing cellulose accessibility is more important than removing lignin: a comparison of cellulose solvent-based lignocellulose fractionation and soaking in aqueous ammonia. Biotechnol. Bioeng. 108:22-30.

Saha BC, Cotta MA (2008). Lime pretreatment, enzymatic saccharification and fermentation of rice hulls to ethanol. Biomass Bioenergy 32:971-977.

Saha BC, Yoshida T, Cotta MA, Sonomoto K (2013). Hydrothermal pretreatment and enzymatic saccharification of corn stover for efficient ethanol production. Ind. Crops Prod. 44:367-372.

Tadesse H, Luque R (2011). Advances on biomass pretreatment using ionic liquids: An overview. Energy Environ. Sci. 4:3913.

Taherzadeh MJ, Karimi K (2007). Acid-based hydrolysis processes for ethanol from lignocellulosic materials: a review. Bioresources 2:472-499.

Tamaki Y, Mazza G (2010). Measurement of structural carbohydrates, lignins, and micro-components of straw and shives: Effects of extractives, particle size and crop species. Ind. Crops Prod. 31:534-541.

Um BH, van Walsum GP (2012). Effect of pretreatment severity on accumulation of major degradation products from dilute acid pretreated corn stover and subsequent inhibition of enzymatic hydrolysis of cellulose. Appl. Biochem. Biotechnol. 168:406-20.

Vancov T, Alston AS, Brown T, McIntosh S (2012). Use of ionic liquids in converting lignocellulosic material to biofuels. Renew. Energy 45:1-6.

Yang L, Cao J, Mao J, Jin Y (2013). Sodium carbonate-sodium sulfite pretreatment for improving the enzymatic hydrolysis of rice straw. Ind. Crops Prod. 43:711-717.

Zhang J, Wang M, Gao M, Fang X, Yano S, Qin S, Xia R (2012).Efficient acetone-butanol-ethanol production from corncob with a new pretreatment technology—wet disk milling. Bio Energy Res. 6:35-43.

Zhang J, Wang Y, Zhang L, Zhang R, Liu G, Cheng G (2014). Understanding changes in cellulose crystalline structure of lignocellulosic biomass during ionic liquid pretreatment by XRD. Bioresour. Technol. 151:402-405.

Zheng Y, Pan Z, Zhang R (2009a). Overview of biomass pretreatment for cellulosic ethanol production. Int. J. Agric. Biol. Eng. 2:51-68.

Zheng YN, Li LZ, Xian M, Ma YJ, Yang JM, Xu X, He DZ (2009b). Problems with the microbial production of butanol. J. Ind. Microbiol. Biotechnol. 36:1127-38.