Hydrolysate-Recycled Liquid Hot Water Pretreatment of Reed Straw and Corn Stover for Bioethanol Production with Fed-Batch, Semi-Simultaneous Saccharification and Fermentation

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Prehydrolysates and water-insoluble solids (WISs) were produced from reed straw and corn stover pretreated with hydrolysate-recycled liquid hot water (LHW) at different cycle times. The chemical components of the prehydrolysates and WISs were then investigated to assess the possible effects of hydrolysate recycling on bioethanol production. The WISs were subjected to fed-batch, semi-simultaneous saccharification and fermentation (S-SSF) to investigate the changes in bioethanol concentration and evaluate the efficiency of the pretreatment. The pretreatment conditions consisted of a temperature of 195 °C, time of 20 min, and liquid ratio of 1:20. The prehydrolysates were recycled using a circulation volume of 50% and were applied to 10 cycles. The results showed that recycling did not significantly decrease the pH of the hydrolysates. The content of glucose and xylan in the hydrolysates decreased and then increased with increasing cycle times. In the WISs, the contents of benzene alcohol extractives and ash increased remarkably. The content of acid-insoluble lignin and glucan increased slightly. The amounts of xylan and acid-soluble lignin in the WISs were low, and the changes in these contents were not significant. Thus, hydrolysate-recycled LHW pretreatment was beneficial for bioethanol production from reed straw, but not from corn stover.

Keywords: Bioethanol; Liquid hot water pretreatment; Hydrolysate-recycled; Reed straw; Corn stover

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INTRODUCTION

Bioethanol is primarily produced from starch materials. However, the limited availability and relatively high price impede large-scale production of bioethanol from starch materials (Bai *et al.* 2007). As the largest source of sugar for fermentation, lignocellulosic biomass is a promising raw material for bioethanol production (Guerriero *et al.* 2016). Therefore, the conversion of lignocellulosic biomass into bioethanol should be investigated for the potential to develop new energy resources (Oberoi *et al.* 2011). Converting lignocellulosic biomass into bioethanol generally involves three steps: pretreatment, enzymatic hydrolysis, and fermentation. Pretreatment is necessary to convert lignocellulosic biomass into bioethanol with high efficiency (Dionisi *et al.* 2015). Therefore, various methods have been developed to improve biomass fractionation through pretreatment substances and processes, such as dilute acid (Hsu *et al.* 2010), alkaline substances (Kim and Han 2012), aqueous ammonia (Gupta and Lee 2010), steam explosion (Yu *et al.* 2012), wet oxidation (Arvaniti *et al.* 2012), bleaching (Pažitný *et al*. 2011), and phosphoric acid-acetone (Kang *et al.* 2012). However, economic and environmental requirements limit the applicability of these methods. Thus, other highly promising pretreatment processes, such as liquid hot water (LHW) (Lu *et al.* 2013b; Ko *et al.* 2015a), have been established for lignocellulosic biomass.

Liquid hot water pretreatment has been recognized as an environmentally friendly technology. A previous study investigated the important characteristics of reed straw and corn stover that determine successful LHW pretreatment. The results revealed that LHW could remove up to 80% of hemicelluloses and enhance enzymatic digestibility (Lu *et al.* 2012). A large amount of water was placed in the system in the prehydrolysis stage, but the concentration of xylan and glucose in the prehydrolysates were very low. As such, they were difficult to reuse. Thus, the researchers considered recycling these prehydrolysates into the system. This procedure could help increase the concentrations of xylan and glucose in the hydrolysates and conserve a large amount of fresh water.

The present study explored the effects of hydrolysate-recycled LHW pretreatment on the changes in the lignin, cellulose, and hemicellulose content in reed straw and corn stover. The compositions of the prehydrolysates and water-insoluble solids (WISs) were analyzed. Fed-batch semi-simultaneous saccharification and fermentation (S-SSF) was performed to evaluate the efficiency of hydrolysate-recycled LHW pretreatment. This method does not affect the conversion rate of cellulose to bioethanol and thus improves the production of bioethanol. This study also aimed to provide a basis for the bioethanol production of reed straw and corn stover through hydrolysate-recycled LHW pretreatment.

EXPERIMENTAL

Materials

Reed straw was provided by Yingkou Papermaking Mill (Yingkou, China). Corn stover was collected from a field near the new district of Jinzhou, Dalian, China. The reed straw and corn stover were milled to particle size of 20-mesh to 80-mesh using laboratory ball mill (Taijihuan Nanometer Limited Company, Qinhuangdao, China) and stored in plastic bag until used. Commercial cellulase was purchased from Imperial JADE Biotechnology Ltd., Co. (Ningxia, China). The *Saccharomyces cerevisiae* was a commercially available type (Angel®) purchased from Angel Yeast Co., Ltd., China.

LHW Pretreatment

The LHW pretreatment was conducted in a 15-L digester with four small tanks (Mechanical Mill of the Shanxi University of Science and Technology, China). The digester was electrically heated and rotated around the motor-driven axis to ensure material uniformity. The material was transferred into the four tanks, which were placed into the digester. Water, as a heat-transfer medium, was poured into the digester to heat the tanks. Approximately 40 g of the materials and 800 mL of de-ionized water were loaded into the small tanks. The pretreatment conditions were as follows: temperature, 195 °C; time, 20 min; and the solid:liquid ratio, 1:20. After pretreatment, the WISs and prehydrolysates were filtered and separated with a Büchner funnel. The prehydrolysates and WISs were stored at 4 °C. After the chemical components of the WISs and prehydrolysates were analyzed, and the prehydrolysates were recycled as pretreatment liquid in the next round. The circulation volume was 50%, and the circulation was repeated 10 times. The WISs were used for the subsequent fed-batch S-SSF.

Fed-Batch S-SSF

The WISs from the hydrolysate-recycled LHW pretreatment experiments were used as substrates. About 5 g of WIS (oven-dried weight) were added into 100-mL Erlenmeyer flasks, with each flask containing a certain amount of pH 4.8 buffer. The enzyme loading was 50 filter paper units per gram of oven-dried WIS, and the medium temperature was maintained at 50 °C during prehydrolysis. After prehydrolysis, the medium temperature was adjusted to 36 °C and maintained throughout the subsequent SSF phase. Afterward, about 1 mL of the activated yeast was added to the medium. The yeast was activated before fermentation. About 1 g of dry yeast was added to 20 mL of 5% sterilized glucose solution. The mixture was activated at 38 °C for 1 h and then cooled between 28 °C and 30 °C before being used in the experiment. The fed-batch S-SSF substrates, which were the preweighed, semi-dried WISs, were added into the Erlenmeyer flasks during the prehydrolysis of fed-batch S-SSF at different prehydrolysis times. The experiments were performed in constant-temperature water-bath shaker (THZ-82, Huafeng Instrument Co., Ltd., Jintan, China) for 72 h. The fermentation time was 72 h. The flasks were sealed with rubber stoppers and equipped with syringe needles to remove the generated carbon dioxide. The samples were collected at 72 h for glucose and ethanol concentration determination.

Analysis Methods

The moisture content of the WIS was determined using GB/T 2677.2 (1993). The sample was dried at the predetermined temperature of 105 °C ± 2 °C until constant mass was achieved. The moisture content was determined as the ratio of the lost quality and the original sample mass and expressed as a percentage. The benzene-alcohol (2:1) extractive contents were determined using the Chinese National Standard methods (GB/T 2677.6 1994). The sample was extracted with the benzene-alcohol mixture (2:1), dried, and then weighed. The acid-insoluble lignin content was determined using Chinese National Standard methods (GB/T 2677.8 1994). The acid-insoluble lignin content was determined as the ratio of the hydrolysis residue mass and the original sample mass. The content of acid-soluble lignin was determined using the method described in GB/T 10337 (1989). The absorption value of the filtrate obtained after separation of the acid-insoluble lignin with 72% sulfuric acid was measured using an ultraviolet (UV) spectrophotometer at 205 nm. The acid-soluble lignin content was calculated from the absorption value. Glucan content was then determined in accordance with the National Renewable Energy Laboratory methods (Sluiter *et al.* 2008). The xylan content was determined using the Chinese National Standard methods (GB/T 2677.9 1994). The samples formed azeotropes with 12% mass fraction of hydrochloric acid to convert pentosan into furfural. The furfural content was measured quantitatively using tetrabromide, and the analytical result was used to calculate the xylan content. Ash content was determined using the Chinese National Standard methods (GB/T 2677.3 1993). The sample organic component was converted into carbon dioxide and water vapor through carbonization and burning at high temperatures. The ash content was determined as the ratio of the quality of the remaining inorganic mass and the original sample mass. Glucose content and ethanol concentration were identified using a SBA-40D Biological Sensing Analyzer (Biology Institute of the Shandong Academy of Sciences, Jinan, China). Glucan content (%) was calculated using Eq. 1,

$$\textit{Glucan content} = (\textit{glucose} \times 0.087 \times 0.9 \times 100\%) / m \qquad (1)$$

where glucose is the glucose concentration (g/L), m is the mass of the oven-dried WIS (g), 0.087 is the volume of the acid hydrolysis liquid (L), and 0.9 is the conversion factor for glucose.

RESULTS AND DISCUSSION

pH Changes in the Prehydrolysates

Liquid water is important for improving the process efficiency during LHW pretreatment (Kou *et al.* 2014). The pH of the LHW-pretreated lignocellulosic biomass was generally within the range of 4 to 5 without the addition of any base or buffer, as the biomass itself is often self-buffering. At high temperatures, liquid water lowers the pH of the reaction environment. The ion product concentration of liquid water at 200 °C is 10^{-11} and attains a pH of 5.6 (Mosier *et al.* 2005). By contrast, the pH value is 7.0 when the water is at 25 °C. The pH of the prehydrolysates after LHW pretreatment was limited to the range between 4 and 7 to minimize the formation of monomeric sugars and sugar decomposition products. The pH changes in the prehydrolysates of corn stover and reed straw in hydrolysate-recycled LHW pretreatment were investigated, and the pH of the hydrolysates before and after pretreatment was determined.

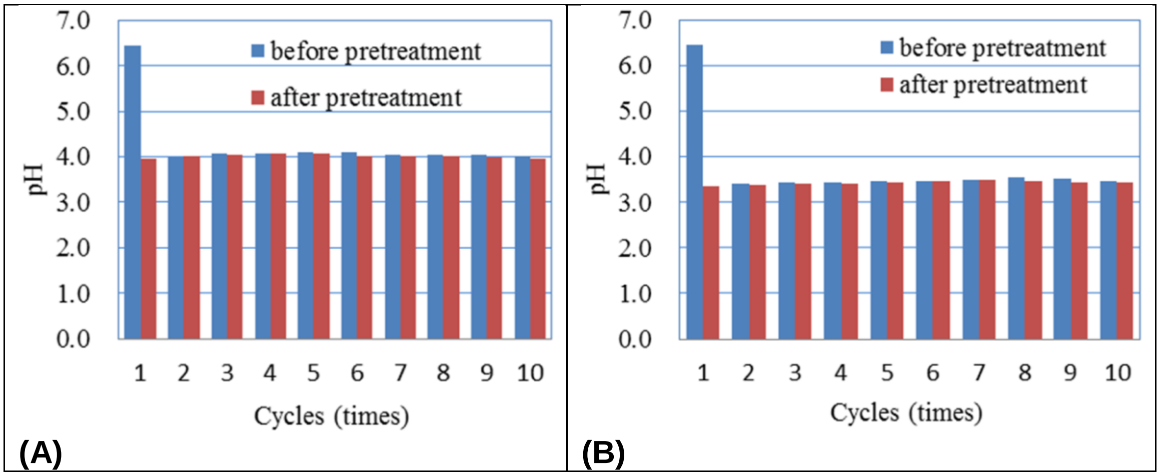

Fig. 1. pH change of hydrolysates during hydrolysate-recycled LHW pretreatment
(A): Corn stover, (B): Reed straw

Figure 1 shows the pH changes in the prehydrolysates of corn stover (A) and reed straw (B) during the hydrolysate-recycled LHW pretreatment process. The same variation trends were achieved. The pH decreased rapidly after the first round of pretreatment, from 6.45 to 3.95 (corn stover) and 3.25 (reed straw). When cycle times were further added, the pH did not change remarkably. These results indicate that the hydrolysate-recycled liquid did not noticeably decrease the pH. The pH was kept stable, but the pH of the hydrolysates of reed straw decreased to less than 4, which could induce the formation of sugar decomposition products. Thus, if the reed straw were used as raw material, the pH would need to be controlled to obtain high bioethanol yield.

Changes in the Glucose and Xylan Content of the Prehydrolysates

The glucose and xylan in the prehydrolysates originated primarily from the hemicelluloses in the lignocellulose biomass. Hemicellulose connects lignin and cellulose and increases the rigidity of the whole cellulose-hemicellulose-lignin network (Donohoe *et al.* 2008). Hemicellulose possesses a lower molecular weight than that of cellulose. Additionally, hemicellulose’s branches contain short, lateral chains that are easily hydrolyzable and consist of different sugars, such as pentoses (xylose and arabinose), hexoses (mannose, glucose, and galactose), and uronic acid (Hendriks and Zeeman 2009). Cellulose, however, is composed of D-glucose subunits linked by β-1,4-glycosidic bonds. These bonds are difficult to break with LHW pretreatment (Galbe and Zacchi 2007). Therefore, the glucose content of the hydrolysates was chiefly derived from the hemicelluloses. The large amount of hemicelluloses was insoluble in the hydrolysates. The hemicelluloses primarily included xylan and glucose. The changes in the glucose and xylan content of the prehydrolysates in the hydrolysate-recycled LHW pretreatment are presented in Figs. 2(A) and (B). Figure 2(A) shows that the glucose concentration in the hydrolysates was highest at the first round. The glucose concentration decreased and then increased when the cycle times increased. Figure 2(B) reveals that the xylan concentration in the hydrolysates was also highest at the first round. The xylan concentration decreased and then increased when the cycle times increased. This effect may have been caused by the generation of inhibitors, such as furfural, during the LHW pretreatment through the degradation of cellulose and hemicelluloses. The formation of these inhibitors may have lowered the yield of fermentable sugars obtained from the pretreated lignocellulosic biomass.

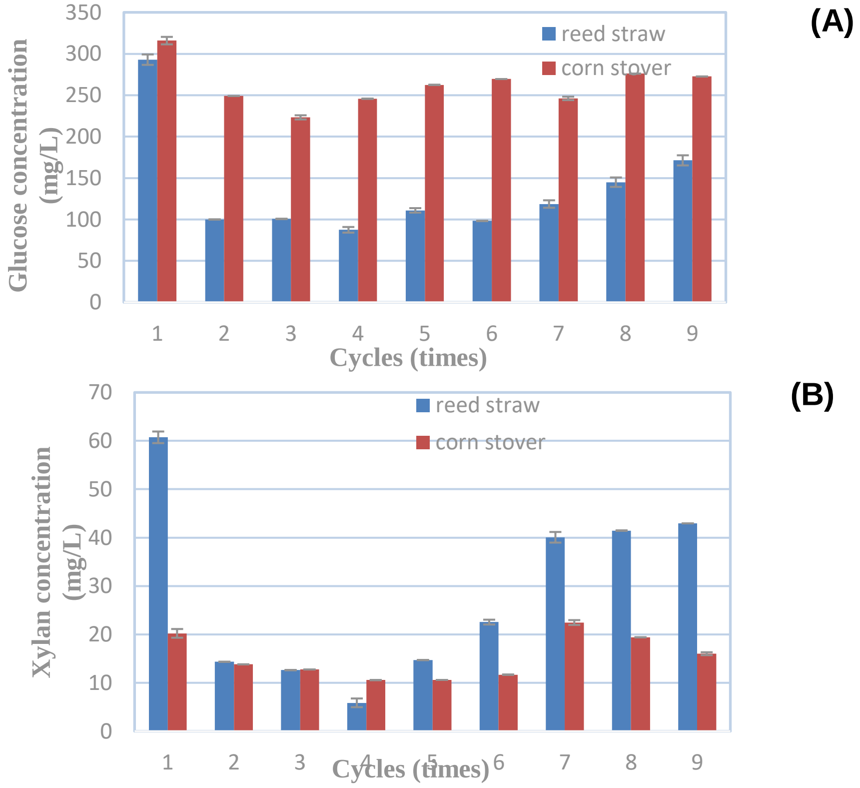

Fig. 2. Changes in glucose (A) and xylan (B) concentration in the hydrolysates during the hydrolysate-recycled LHW pretreatment

Changes in the Benzene-Alcohol Extractive Contents of the WISs

During LHW pretreatment, the water extracts were composed primarily of monosaccharides, a small amount of oligosaccharides, and water-soluble polysaccharides (Lu *et al.* 2016). However, some substances could not be extracted with water, such as the terpene compounds, fats, waxes, and tannins. These substances remained as main components of the insoluble solids. However, these substances could be extracted with benzene-alcohol. The main benzene-alcohol extractives were waxes, as well as small amounts of the large fatty acids (Lu *et al.* 2014). Therefore, the pretreatment produced "clean" cellulose, which facilitates enzymatic hydrolysis and yeast fermentation. The changes in the benzene-alcohol extractable content of the WISs in the hydrolysate-recycled LHW pretreatment are shown in Fig. 3. The benzene-alcohol extractable content of reed straw changed slightly, with an increase of the pretreatment cycle time, but the content was below 10%. Furthermore, the number of cycles did not correlate with the benzene-alcohol extractable content. In the corn stover, the benzene-alcohol extractable content initially increased with increasing cycle number. The benzene-alcohol extractable content was about 10% to 25%, which is very high. In the third cycle, the content reached the maximum value of 25%, and then decreased and gradually stabilized at 20%. Certain extracted substances likely underwent a degradation reaction in the highly acidic conditions.

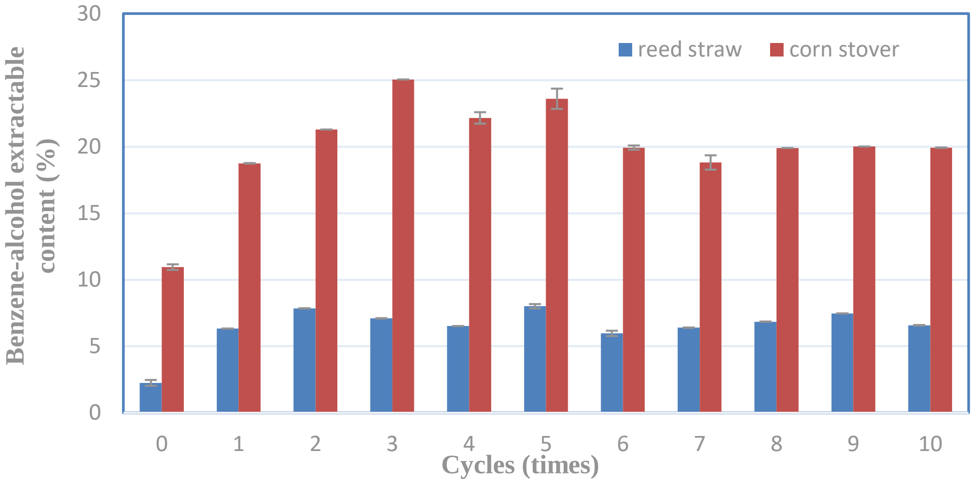

Fig. 3. Changes in the benzene-alcohol extractable content in the WISs of the hydrolysate-recycled LHW pretreatment

Changes in the Acid-Insoluble and Acid-Soluble Lignin Content of the WISs

Lignin is an amorphous heteropolymer consisting of three different phenylpropane units: guiacyl, syringyl, and p-hydroxyphenyl (Tan *et al.* 2015). Because of its structure, lignin is generally a critical resistance factor against the enzymatic hydrolysis of cellulose (Alvira *et al.* 2010). The glucose yield from enzymatic digestion was greatly improved when lignin was removed or degraded during pretreatment (Guo *et al.* 2014). However, a large amount of lignin was retained in the WISs during LHW pretreatment. The changes in the acid-insoluble and acid-soluble lignin contents of the WISs in the hydrolysate-recycled LHW pretreatment are shown in Fig. 4. As shown in Fig. 4(A), the acid-insoluble lignin content of corn stover initially increased with increasing cycle time, and then decreased, before finally stabilizing. The maximum acid-insoluble lignin content was reached in the fourth round, but a stable state was achieved at the end. The acid-insoluble lignin content of reed straw generally increased with the increase with the number of cycles. Figure 4(B) shows the changes in acid-soluble lignin content, which were not obvious with increasing cycle number. These results could be explained by the higher reaction affinity of carbohydrate with hot water than that of lignin during pretreatment. The lignin fraction of the lignocellulosic materials can be partly depolymerized in water during autohydrolysis and then converted into phenolic compounds. This observation implies that the structure of lignin in the WISs, along with the pretreatment conditions, may have affected the enzymatic digestibility.

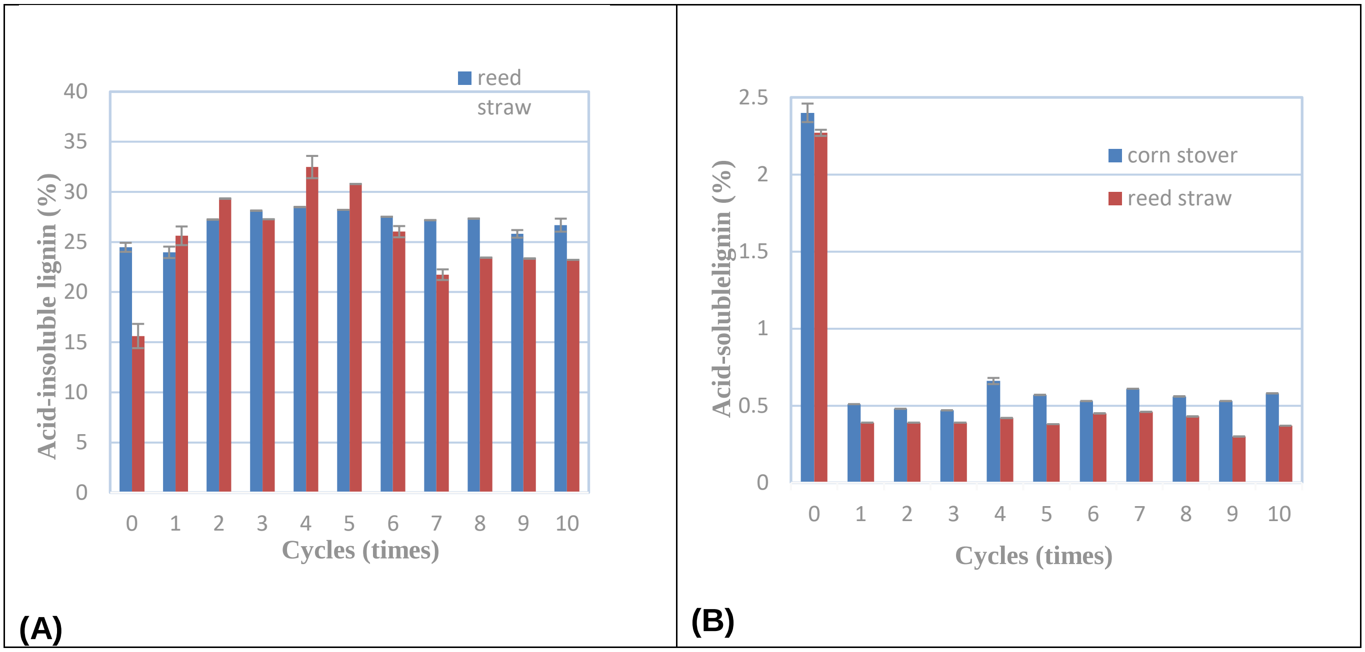

Fig. 4. Changes in the acid-insoluble (A) and acid-soluble (B) lignin content in the WISs of the circulating prehydrolysate LHW pretreatment

Changes in the Glucan and Xylan Content of the WISs

The presence of hemicellulose hinders the contact between cellulase and cellulose, inhibiting the enzymatic reaction. However, the hemicelluloses would have been removed in the LHW pretreatment (Ko *et al.* 2015b). Cellulose is the main carbohydrate source for hydrolysis and fermentation to ethanol. Therefore, avoiding cellulose solubilization is a key determinant of pretreatment efficiency, and it is achieved by providing numerous cellulose residues after pretreatment. The glucan and xylan content of the WISs were important factors. The changes in the glucan and xylan content of the WISs of the hydrolysate-recycled LHW pretreatment are shown in Figs. 5(A), (B). Figure 5(A) shows that the glucan content of reed straw was slightly higher than that of corn stover. However, with increasing cycle number, the changes in glucan content of the corn stover were not apparent. Figure 5(B) reveals that the xylan content was decreased substantially after LHW pretreatment, which may have been the result of the hot-water-induced cleavage of hemicellulose linkages and liberation of o-acetyl and other acid moieties from hemicelluloses, thereby forming organic acids. The release of these acids catalyzes the removal of oligosaccharides from hemicelluloses.

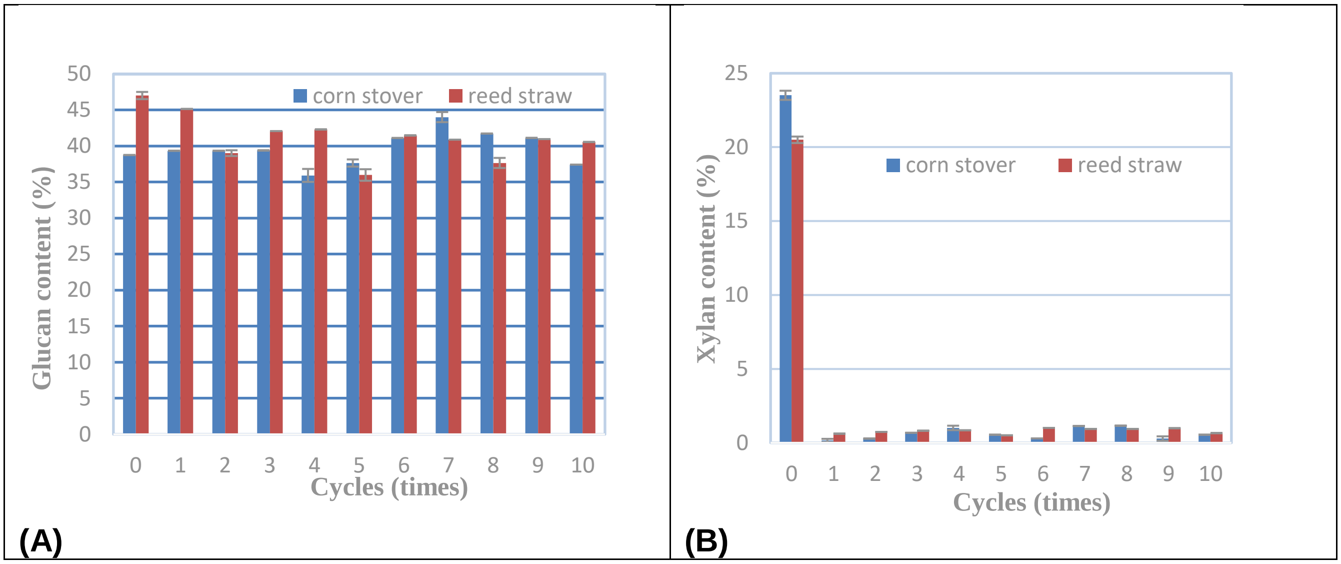

Fig. 5. Changes in glucan (A) and xylan (B) content in the WISs of the hydrolysate-recycled LHW pretreatment

Change in the Ash Content of the WISs

Ash in lignocellulosic biomass is usually composed of oxide mineral elements. Although only a small amount of ash is present in lignocellulosic biomass, and 60% of the ash consists of SiO_2. During the hydrolysate-recycled LHW pretreatment, a portion of the inorganic salt in the lignocellulosic biomass and the extracted glucan, xylan, pectin, and starch became dissolved in the hot water. Thus, the ash content of the different lignocellulosic biomasses changed. The changes in the ash content of the WISs in the hydrolysate-recycled LHW pretreatment are illustrated in Fig. 6. The ash content in the WISs of reed straw was higher than that in the WISs of corn stover (Fig. 6). The ash content in the WISs from reed straw increased with increasing cycle time, but the ash content in the WISs from corn stover decreased as the number of cycles increased. This effect may have been the result of increased sugar dissolution, but the effect of ash on enzymatic hydrolysis and fermentation remains unknown.

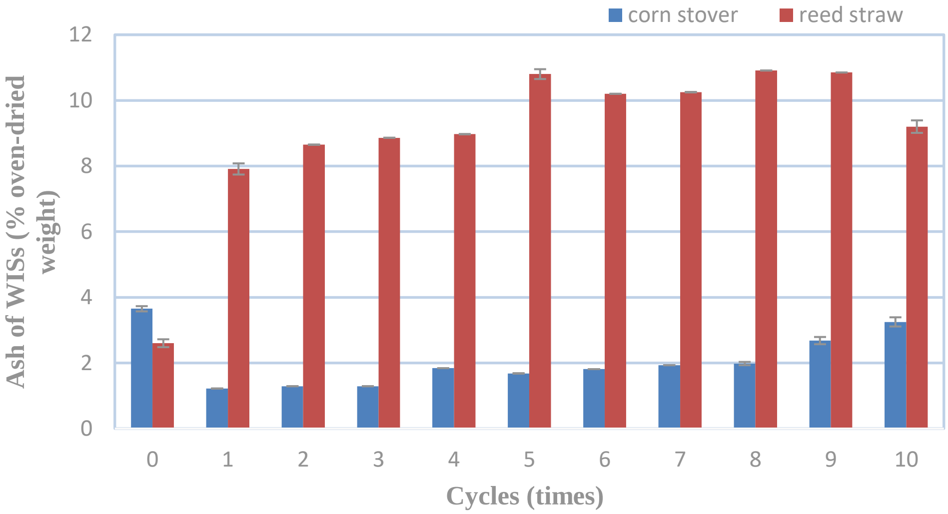

Fig. 6. Changes in the ash content of the WISs in the hydrolysate-recycled LHW pretreatment

Fed-Batch S-SSF of WISs after Hydrolysate-Recycled LHW Pretreatment

Several studies have evaluated the importance of using fed-batch methods in S-SSF to increase bioethanol concentration (Shen and Agblevor 2010; Lu *et al.* 2013a; Buruiana *et al.* 2014; Li *et al.* 2014). Fed-batch S-SSF was used in this study to assess the effect of recycled hydrolysates on bioethanol concentration. The bioethanol concentration in the fed-batch S-SSF of WISs after hydrolysate-recycled LHW pretreatment are presented in Fig. 7. However, the bioethanol concentration of corn stover was noticeably different from that of reed straw. The highest amount of bioethanol produced by reed straw was obtained with six cycles. This result indicated that appropriate recycling of hydrolysates was beneficial for the fed-batch S-SSF of the reed straw. Conversely, the amount of bioethanol produced from corn stover was generally reduced when the number of cycles was increased. This finding suggested that the recycled hydrolysates were not beneficial for bioethanol production from corn stover. Therefore, different biomass materials exhibited different characteristics in the fed-batch S-SSF after hydrolysate-recycled LHW pretreatment was performed.

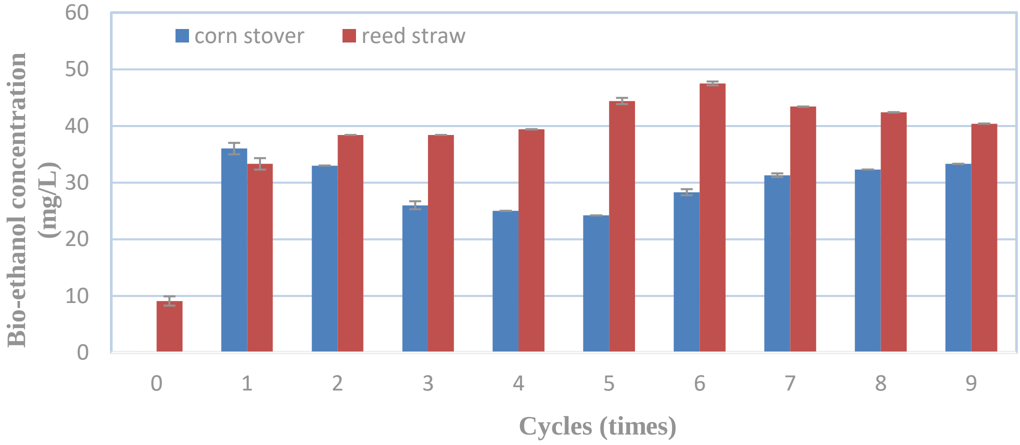

Fig. 7. Fed-batch S-SSF of the WISs pretreated by the hydrolysate-recycled LHW

CONCLUSIONS

1. Hydrolysate from hot water pretreatment of reed straw and corn stover was used multiple times, with a recycle rate of 50%. Such recycling did not remarkably decrease the pH of the hydrolysates. The content of glucose and xylan in the hydrolysates decreased and then increased with increasing cycles.
2. In the WISs, the content of benzene-alcohol extractives and ash increased greatly. The content of acid-insoluble lignin and glucan also increased slightly. The amounts of xylan and acid-soluble lignin in the WISs were low, and there were no major changes in the content.
3. Hydrolysate-recycled LHW pretreatment was beneficial for bioethanol production from reed straw, but it was not beneficial for bioethanol production from corn stover. Different lignocellulosic biomasses exhibited different characteristics of fed-batch S-SSF after hydrolysate-recycled LHW pretreatment was performed.

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