Bioresource Technology 199 (2016) 228–234

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Biobutanol production from corn stover hydrolysate pretreated with recycled ionic liquid by *Clostridium saccharobutylicum* DSM 13864

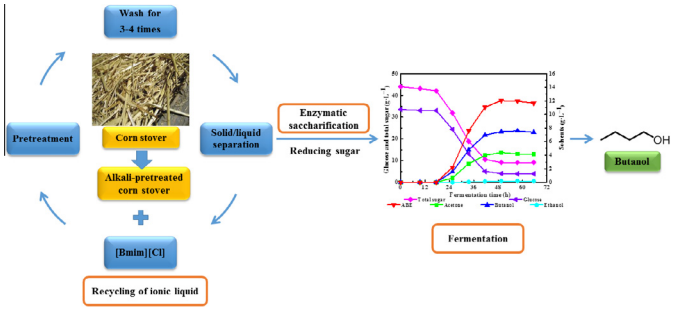
Ji-Cai Ding, Guo-Chao Xu, Rui-Zhi Han, Ye Ni *

The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, Jiangsu, China

HIGHLIGHTS

- Corn stover (CS) was pretreated by alkali (NaOH) followed by ionic liquid [Bmim][Cl].
- [Bmim][Cl] can be recycled for at least ten times without obvious loss of efficiency.
- CS hydrolysate pretreated by recycled [Bmim][Cl] was successfully used in butanol fermentation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 22 June 2015
Received in revised form 28 July 2015
Accepted 30 July 2015
Available online 20 August 2015

Keywords:
Corn stover
Pretreatment
[Bmim][Cl]
Ionic liquid
Butanol fermentation

ABSTRACT

In this study, corn stover (CS) hydrolysates, pretreated by fresh and recycled ionic liquid (IL) [Bmim][Cl], were utilized in butanol fermentation by *Clostridium saccharobutylicum* DSM 13864. An efficient CS pretreatment procedure using [Bmim][Cl] was developed, giving a glucose concentration of 18.7 g L^{-1} using ten times recycled [Bmim][Cl], representing about 77% of that produced with fresh IL (24.2 g L^{-1}). Fermentation of hydrolysate I (pretreated by fresh IL) resulted in 7.4 g L^{-1} butanol with a yield of 0.21 g $g^{-1}_{total\text{-}sugar}$ and a productivity of 0.11 g L^{-1} h^{-1}, while 7.9 g L^{-1} butanol was achieved in fermentation using hydrolysate II (pretreated by ten times reused IL) with similar levels of acetone and ethanol, as well as yield and productivity. This study provides evidence for the efficient utilization of IL in CS pretreatment for biobutanol fermentation.

1. Introduction

With the limited supply of fossil fuels and the rise of the oil price, there have been renewed interest and increased effort toward the biofuels production through fermentation of lignocellulosic biomass (Demirbas, 2008; Galbe and Zacchi, 2007). Acetone–butanol–ethanol (ABE) fermentation ranks the second largest biotechnological process ever performed for the production of biofuels, which has been maintained for several decades in China (Ni and Sun, 2009). Biobutanol, a major product of ABE fermentation, is an excellent feedstock chemical, as solvents for hormones, drugs, antibiotics, cosmetics, hydraulic fluids, and vitamins production (Qureshi et al., 2010a; Lee et al., 2008). In recent years, biobutanol emerged as a direct replacement of gasoline or as a fuel additive. Compared with ethanol, butanol is particular attractive for application as a biofuel due to its superior chemical and physical features such as higher energy density (29.2 MJL^{-1}), lower viscosity, lower tendency to absorb water and less corrosive for certain motor parts (Ni et al., 2012).

A number of researches have been reported in the biological formation of butanol since the first fermentation by Pasteur in 1862. However, butanol fermentation in large-scale has been severely hindered by its high substrate cost, low product yield,

* Corresponding author.
E-mail address: yni@jiangnan.edu.cn (Y. Ni).

http://dx.doi.org/10.1016/j.biortech.2015.07.119
0960-8524/

and high recovery cost (Qureshi et al., 2001). In order to overcome these hurdles, researchers have committed many efforts such as using inexpensive substrates, developing microbial hyper producing strains, and optimizing fermentation conditions (Li et al., 2013). Recently, fermentable sugars converted from lignocellulosic biomass, an abundant and renewable carbon source, are considered to be worthy and promising substrates for butanol production. Butanol can be produced from various lignocellulosic substrates through pretreatment, enzymatic hydrolysis and fermentation, including corn stover (CS) (Lloyd, 1984), wheat straw (Qureshi et al., 2007), corn fiber (Qureshi et al., 2008), distillers dry grain solubles (Ezeji and Blaschek, 2008), barley straw (Qureshi et al., 2010a), CS and switchgrass (Qureshi et al., 2010b) as well as sweet sorghum bagasse (Zhang et al., 2011). The most favorable lignocellulosic biomass used for butanol production should have high cellulose and hemicellulose content as well as low lignin content, and hence barley straw was considered to be the right choice (Baral et al., 2014). However, for agricultural countries especially China, CS is a more suitable biomass because of its large quantities, low costs and high content of cellulose.

Lignocellulosic materials mainly consist of cellulose (40–60%), hemicellulose (20–40%), and lignin (10–24%) (Geng and Henderson, 2012). Cellulose and hemicellulose can be converted into simple sugars and further to biofuels and other chemicals through fermentation processes. Lignin has been regarded as the main barrier for the enzymatic digestion of biomass because it can hinder enzymatic hydrolysis by unproductive adsorption of cellulose, and impeding enzymatic accessibility to cellulose and hemicellulose (Kim et al., 2003; Yang and Wyman, 2004). To achieve higher sugar concentrations from lignocellulosic biomass, a number of pretreatment processes have been developed, such as dilute acid, alkali extraction, steam explosion, and organic solvent extraction, through reducing the crystallinity as well as the degree of polymerization of cellulose and increasing the porosity of the lignocellulosic materials (Chum et al., 1988; MacDonald et al., 1983; Saha et al., 2005; Schell et al., 1992). However, a cost-effective biofuel production process requires minimized downstream inhibitors, low processing cost, and easy recycling (Geng and Henderson, 2012). Adoption of ionic liquids (ILs) as solvents to pretreat lignocellulosic biomass has received great attention during the last decade (Kosan et al., 2007). ILs pretreatment is considered to be able to reduce cellulose crystallinity, hemicellulose and lignin content of biomass, and increase its surface area, enzymatic hydrolysis kinetics, and the yield of fermentable sugars (Li et al., 2010; Samayam and Schall, 2010). In addition, ILs have many advantages including low melting points, wide liquid temperature range, high polarities, high thermal and chemical stability, non-flammability, negligible vapor pressure, consisting of ions (cations and anions) and good solvating properties (Zavrel et al., 2009; Behera et al., 2014). Among ILs, [Bmim][Cl] was proved to be efficient in pretreatment. Sathitsuksanoh et al. used [Bmim][Cl] to pretreat corn stover and found that 55% glucan digestibility was reached after 72 h of enzymatic hydrolysis of [Bmim][Cl] pretreated corn stover, which was about 2.5-fold compared with untreated corn stover (Sathitsuksanoh et al., 2012).

Based on our previous study on sugars (glucose, fructose, sucrose, xylose, and arabinose) utilization by various *Clostridial* strains, *Clostridium saccharobutylicum* DSM 13864 showed the highest solvent producing efficiency, and could be used for butanol fermentation from both cane molasses and lignocellulosic hydrolysates (Ni et al., 2013). Here, butanol production from lignocellulosic biomass (such as corn stover) by this strain was investigated. However, most of butanol fermentation processes have been carried out using dilute acid or steam explosion pretreated lignocellulosic biomass, which usually introduce inhibitors for the growth of *Clostridial* strains. Since lignin is a highly cross-linked aromatic polymer and can hinder enzymatic hydrolysis, we proposed using the combination of alkali (NaOH) and IL [Bmim][Cl] to remove the lignin and improve the concentration of fermentable sugars. The effectiveness of the recycled IL was investigated for reduced cost, and the CS hydrolysates pretreated by recycled IL were also attempted in butanol fermentation.

2. Methods

2.1. Strains, biomass, and chemical

C. saccharobutylicum DSM 13864 was purchased from DSMZ. It was cultivated in Reinforced Clostridia Medium (RCM) at 37 °C for 7 days to induce sporulation, followed by storage at room temperature. Spores suspension (10%, v/v) was inoculated into a test tube (Φ1.5 cm × 15 cm) containing 12 mL sterile RCM and then placed in a desiccator pumped to a vacuum level of 0.065 MPa for providing an anaerobic condition. Afterwards, the culture was maintained at 37 °C for 12–18 h and supplied as the seed medium (Ni et al., 2013).

CS was purchased from Shandong Zesheng Bioengineering Technology Co., Ltd. The knife-milled CS was passed through a 380 μm sieve and dried at 60 °C for 24 h before use. The particle size distribution of the CS was determined to be 40–44% of 180–380 μm, 37–40% of 120–180 μm, and 19–20% of under 120 μm in diameter.

IL 1-butyl-3-methylimidazolium chloride ([Bmim][Cl], 99%) was purchased from Henan Lihua Pharmaceutical Co., Ltd. ACCELLERASE® 1500 cellulase was a generous gift from Genencor (Wuxi, China) Bio-Products Co., Ltd. The total cellulase activity was determined by the standard filter paper assay using Whatman No. 1 filter paper strip. One unit of enzyme activity (FPU) is defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute at pH 4.8 and 50 °C. The activity was determined to be 60 FPU/mL. All other chemicals purchased from Sinopharm Chemical Reagent Co., Ltd. were of reagent or analytical grade.

2.2. Alkali pretreatment of CS

Twenty grams of dried untreated corn stover (U-CS) (4.2 wt% moisture) was soaked in 300 mL of 1 wt% NaOH at room temperature (20–25 °C) for 1, 2, 3, 4, 5, 6 and 7 days, respectively. The soaked CS was filtered and washed with tap water until pH 7, then washed once with deionized water. The resultant CS was dried at 60 °C for 24 h in an oven. The CS pretreated by alkali is defined as alkali-pretreated corn stover (A-CS). The A-CS was stocked in sealed plastic bags and dried again before use. In the following experiments using combination of IL and alkali pretreatment, the application of IL pretreatment was conducted only on day 1 sample of A-CS.

2.3. IL pretreatment of CS

The IL (100 g) was heated at 130 °C for over 30 min to remove the moisture and then mixed with U-CS and A-CS at a solid:solid ratio of 20:1, respectively. The mixtures were incubated at 130 °C for 2 h with mechanical agitation at 30–60 rpm. The dissolved CS mixtures were poured into 500 mL hot deionized water (about 85 °C) with rapid stirring to regenerate the materials (Geng and Henderson, 2012). The regenerated CS was collected with vacuum filtration, and washed with hot deionized water (about 85 °C) twice to remove the remaining IL. The CS and A-CS pretreated by IL were named as IL-CS and A-IL-CS, respectively. The IL-CS and A-IL-CS were stocked in sealed plastic bags and dried again before use.

2.4. Analysis of CS composition

The components of the cellulose, lignin and ash in U-CS, A-CS, IL-CS and A-IL-CS were analyzed using NREL Laboratory Analytical Procedure (LAP) (Sluiter et al., 2008). The dried CS (300.0 ± 1.0 mg) was hydrolyzed with 72% H_2SO_4 (3.00 ± 0.01 mL, w/w) in a water bath (30 ± 3 °C) for 60 ± 5 min. The mixture was added with 84.00 ± 0.04 mL deionized water to dilute the acid to 4% (w/w) and autoclaved at 121 °C for 1 h. The hydrolyzed samples were filtered using quantitative filter papers (30–50 μm of pore diameter, 0.01% of ash) with a vacuum pump to separate the liquid from the mixture. The solid was used to measure the acid-insoluble lignin and ash content, and the filtrate was used to determine the acid-soluble lignin and monosaccharide content. The concentration of the monosaccharides was determined using a Hitachi High Performance Liquid Chromatography (HPLC) equipped with an Aminex HPX-87H column at 60 °C with 5 mM H_2SO_4 as eluent and a flow rate of 0.5 mL/min.

2.5. Enzymatic hydrolysis of CS

The U-CS, A-CS, IL-CS and A-IL-CS (1.000 ± 0.005 g) were soaked in citrate buffer (50 mM, pH 4.8) in 50-mL conical flasks with plugs for 24 h to assure sufficient penetration of the liquid into the biomass. Cellulase (30 FPU $g^{-1}_{total\text{-}solid}$) was added to the mixture to reach a final solid:liquid ratio of up to 1:15. The mixture was further incubated in a water bath at 50 °C and 120 rpm for 10 h. Samples (0.3 mL) were taken at 2, 4, 6, 8, 10 h, and then subjected to centrifugation at 12,396×*g* for 8 min. The resultant supernatants (200 μL) were mixed with 0.4% H_2SO_4 (800 μL, w/w) to terminate the hydrolysis reaction. The glucose concentration was determined using HPLC analysis as above mentioned. The glucose yield was calculated by dividing the actual glucose obtained in hydrolysis process by theoretical glucose converted from cellulose.

2.6. Optimization of cellulase dosage

The A-IL-CS (0.500 ± 0.005 g) were soaked in citrate buffer (50 mM, pH 4.8) in 50-mL conical flasks with plugs for 24 h. Cellulase was added into the mixtures at dosages of 10, 30, 50 and 70 FPU $g^{-1}_{total\text{-}solid}$ respectively. The final solid:liquid ratio was 1:12 and the mixtures were further kept in a water bath at 50 °C and 120 rpm for 12 h. Samples (100 μL) were collected at 2, 4, 6, 8, 10, 12 h and centrifuged at 12,396×*g* for 8 min. Supernatants (50 μL) were added with 0.4% H_2SO_4 (450 μL, w/w) to stop the hydrolysis reactions. The glucose concentration was determined using HPLC analysis as above described.

2.7. Pretreatment of CS by recycled IL (RIL)

IL (400 g) was added into a 1-L three-necked flask and heated at 130 °C for over 30 min to remove the moisture. Then 20.0 g A-CS was added to the three-necked flask and incubated at 130 °C for 2 h with mechanical agitation. Hot deionized water (800 mL, about 85 °C) was added into the mixture with rapid stirring to regenerate the materials. The regenerated CS was collected with filtration under vacuum and washed for three times with 2.4 L hot deionized water to remove the residual IL. All the filtrates were collected to recycle the IL with rotary evaporator at 80 °C under vacuum, which was sufficient to remove almost all the water from the IL. Then, the recycled ionic liquid (RIL) was used to treat A-CS and the treatment procedure was the same as mentioned above. The A-CS treated by RIL is defined as A-RIL-CS.

2.8. Enzymatic hydrolysis of A-RIL-CS

The A-RIL-CS (1.000 ± 0.005 g) was soaked in citrate buffer (50 mM, pH 4.8) in a 50-mL conical flask with plug for 24 h. Cellulase (50 FPU $g^{-1}_{total\text{-}solid}$) was added to the mixture and the final solid:liquid ratio was 1:20. The A-RIL-CS was fluffy, and was soaked sufficiently at solid:liquid ratio of 1:20. The hydrolysis procedure of A-RIL-CS and detection procedure of glucose concentration were carried out as mentioned above.

2.9. Butanol fermentation

Butanol fermentation was conducted using A-IL-CS hydrolysate and A-RIL-CS hydrolysate, respectively. A-IL-CS hydrolysate (Hydrolysate I, 150 mL) was obtained by hydrolyzing the A-IL-CS in a water bath at 50 °C and 120 rpm for 12 h. The glucose fermentation medium containing 54 g L^{-1} glucose (Glucose I) was used as control. A-RIL-CS hydrolysate (Hydrolysate II) was obtained by hydrolyzing the A-CS pretreated by ten times recycled [Bmim][Cl] in a water bath at 50 °C and 120 rpm for 12 h. Then, the resulted hydrolysate was concentrated to 50 mL to reach a sugar concentration of 50 g L^{-1} before used in fermentation. The glucose fermentation medium containing 50 g L^{-1} glucose (Glucose II) was used as control. The hydrolysate medium was prepared by mixing hydrolysate with other medium components (10 g L^{-1} corn starch powder, 4 g L^{-1} $CaCO_3$, 2 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} K_2HPO_4, 0.01 g L^{-1} $MnSO_4{\cdot}H_2O$). The glucose fermentation medium (as control) contains (g L^{-1}): glucose 54 (Glucose I), 50 (Glucose II); corn starch powder, 10; $CaCO_3$, 4; $(NH_4)_2SO_4$, 2; K_2HPO_4, 0.5; $MnSO_4{\cdot}H_2O$, 0.01. The pH was adjusted to 6.5 with 4 M NaOH, and the medium was sterilized at 115 °C for 20 min.

For Hydrolysate I, the fermentation was conducted in a 250-mL conical flask with 8% of actively growing culture as inoculum followed by incubation at 37 °C in a desiccator (0.065 MPa). After inoculation, the total sugars of Glucose I and Hydrolysate I were determined to be 45.9 and 44.1 g L^{-1}, respectively. For Hydrolysate II, the fermentation was conducted in a 100-mL anaerobic bottle with 20% of actively growing culture as inoculum followed by incubation at 37 °C in a desiccator (0.065 MPa). After inoculation, the total sugars of Glucose II and Hydrolysate II were determined to be 43.7 and 40.1 g L^{-1}, respectively.

Samples were taken intermittently and the ABE contents were analyzed by gas chromatography (6890N; Agilent Technologies, Wilmington, DE, USA) equipped with flame ionization detector (FID) and a capillary column PEG-20 M (30 m × 0.32 mm × 0.5 μm, JK, China) using nitrogen as the carrier gas. The oven temperature was maintained at 60 °C for 0.5 min and then programmed with the increment of 10 °C to 120 °C, held for 0.5 min, and subsequently increased to 190 °C with the increment of 15 °C with 1 min final hold. The temperature of the injector and detector was held at 180 °C and 210 °C, respectively. The injection volume was 1 μL (Ni et al., 2013).

3. Results and discussion

3.1. Pretreatment of corn stover using alkali

Alkali pretreatment is the most widely used method to enhance the enzymatic hydrolysis of various lignocellulosic biomass, due to its outstanding delignification capacity, low toxicity and little formation of inhibiting compounds. However, most of alkali pretreatment methods have been conducted at high temperatures of 90–120 °C or even higher (Geng and Henderson, 2012; Ouajai and Shanks, 2005), which were highly energy-consuming. In order to reduce energy consumption and alkali corrosion, room

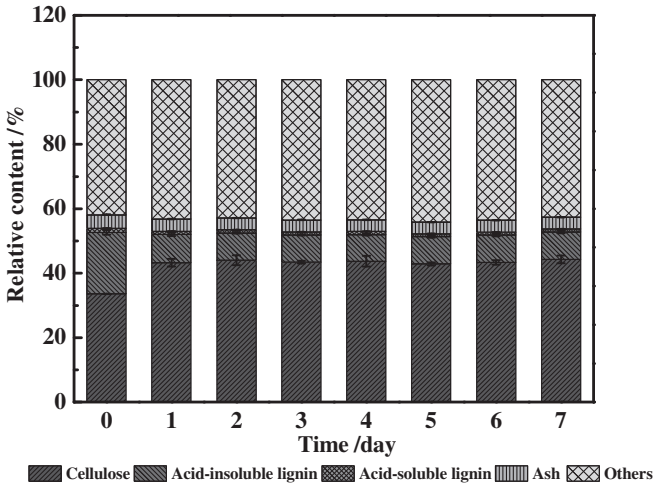


Fig. 1. Chemical composition of the corn stover pretreated by alkali. 0: untreated corn stover; 1–7: corn stover that pretreated by NaOH for 1–7 days. The alkali pretreatment was conducted at 20–25 °C without agitation. The error bars indicate the standard deviation from three independent experiments.

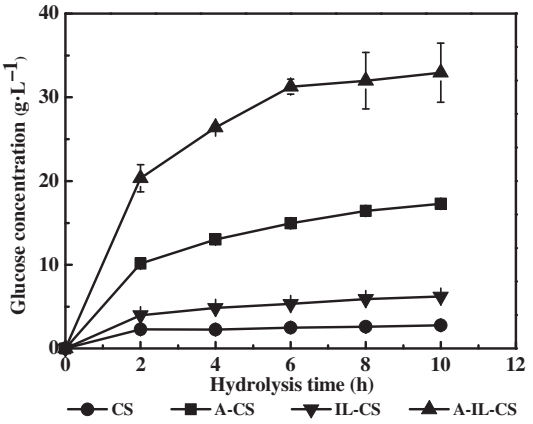


Fig. 2. Glucose concentration after enzymatic hydrolysis of U-CS, A-CS, IL-CS and A-IL-CS. CS: corn stover; A-CS: corn stover pretreated by alkali for 1 day; IL-CS: corn stover pretreated by [Bmim][Cl]; A-IL-CS: corn stover pretreated by alkali and [Bmim][Cl]. The glucose concentration was obtained at a cellulase dosage of 30 FPU $g^{-1}_{total\text{-}solid}$ and a final solid:liquid ratio of 1:15. The error bars indicate the standard deviation from three independent experiments.

temperature and low alkali concentration (1 wt% NaOH) are considered to be feasible in CS pretreatment. Alkali pretreatment reactions were performed without agitation for 1–7 days at room temperature. The chemical compositions (including cellulose, lignin and ash percentages) of U-CS and A-CS were determined as shown in Fig. 1. For U-CS, the contents of cellulose, total lignin and ash were 33.6%, 20.3% and 4.2% respectively, while lignin and ash accounted up about 25% of total weight. After the CS was pretreated by alkali for 7 days, the cellulose percentage was increased by 30%. With regard to total lignin, the amount was reduced by more than 50%, among which the acid-insoluble lignin was reduced from 19.1% (U-CS) to 8.5%, indicating the effectiveness of alkali in the removal of lignin. It is reported that dilute NaOH might cause lignocellulosic biomass to swell, which leads to an increase in the internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure. The ash contents, mainly made up of metal ions and oxides, were reduced about 10% after alkali pretreatment. No obvious promotion effect was detected when extending the pretreatment times from 1 to 7 days (Fig. 1). Therefore, 1 day was selected for dilute NaOH pretreatment in further experiments. Existence of lignin may adsorb cellulases and impede enzymatic accessibility to cellulose and hemicellulose (Nasirpour et al., 2014), therefore deconstruction of lignin helps to enhance the enzymatic hydrolysis (Lee et al., 2009; Geng and Henderson, 2012).

3.2. Effect of ionic liquid pretreatment on U-CS and A-CS

[Bmim][Cl] have been proven to be one of the most intensively studied ILs in the pretreatment of lignocellulosic biomass for biofuels production. The effect of ionic liquid and its combination with dilute alkali pretreatments on the CS were investigated. U-CS and A-CS were incubated with [Bmim][Cl] at 130 °C for 2 h to generate IL-CS and A-IL-CS. Chemical compositions of IL-CS, A-IL-CS were analyzed as tabulated in Table 1. IL pretreatment could increase the cellulose content from 33.6% (U-CS) to 41.6% in IL-CS, and further improved to 54.4% after combination with alkali treatment, indicating [Bmim][Cl] is efficient in the pretreatment of CS. With regard to lignin, however, little effect was found in the case of [Bmim][Cl] pretreatment. When combining with alkali pretreatment, the lignin was decreased to 8.6% in A-IL-CS, representing a 58% decrease in lignin than that in U-CS. ILs have been considered to be able to change the structure of cellulose and dissolve much of the lignin (Cetinkol et al., 2010). Whereas when water or other precipitants (such as ethanol and acetone) were added into the IL-dissolved materials, most of the lignin will be recovered (Geng and Henderson, 2012). However, addition of dilute NaOH could avoid the recovery of lignin. Consequently, in this study, before IL treatment, dilute NaOH pretreatment was carried out to reduce the lignin content. It is also interesting to note that IL could also reduce the ash from 4.2% (U-CS) to 3.0% (IL-CS) and 2.8% (A-IL-CS).

3.3. Optimization of enzymatic hydrolysis

After deconstruction of lignin from lignocellulosic biomass, cellulase was introduced to further convert biomass into reducing sugars, mainly glucose, which could be used as carbon sources for biobutanol production. U-CS, A-CS, IL-CS and A-IL-CS were hydrolyzed using ACCELLERASE® 1500 cellulase at 30 FPU $g^{-1}_{total\text{-}solid}$. The glucose concentrations after enzymatic saccharification were illustrated in Fig. 2. Glucose of about 33 g L^{-1} was released from A-IL-CS

Table 1
Chemical composition of CS, A-CS, IL-CS and A-IL-CS.

	Cellulose (%)	Acid-soluble lignin (%)	Acid-insoluble lignin (%)	Ash (%)	Others (%)
CS	33.6 ± 0.0	1.2 ± 0.0	19.1 ± 0.7	4.2 ± 0.1	41.9
A-CS	43.2 ± 1.2	1.0 ± 0.0	8.8 ± 0.5	3.8 ± 0.1	43.2
IL-CS	41.6 ± 1.1	1.0 ± 0.0	17.5 ± 1.2	3.0 ± 0.1	36.9
A-IL-CS	54.4 ± 0.6	0.9 ± 0.0	7.7 ± 0.9	2.8 ± 0.4	34.2

CS: corn stover; A-CS: corn stover pretreated by alkali for 1 day; IL-CS: corn stover pretreated by [Bmim][Cl]; A-IL-CS: corn stover pretreated by alkali and [Bmim][Cl]. The data were expressed as mean ± standard error from three independent experiments.

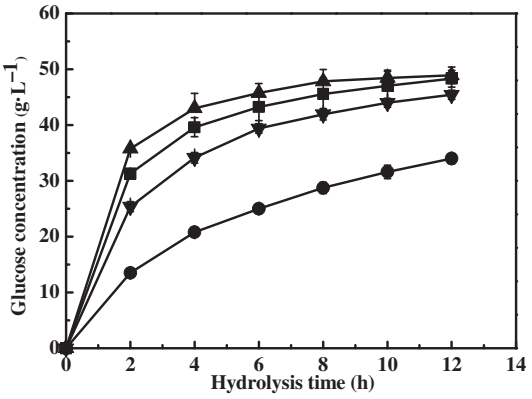


Fig. 3. Optimization of cellulase dosages in the enzymatic saccharification of A-IL-CS. (●) 10 FPU $g^{-1}_{total\text{-}solid}$; (▼) 30 FPU $g^{-1}_{total\text{-}solid}$; (■) 50 FPU $g^{-1}_{total\text{-}solid}$; (▲) 70 FPU $g^{-1}_{total\text{-}solid}$. A-IL-CS: corn stover pretreated by alkali and [Bmim][Cl]. The glucose concentration was obtained at different cellulase dosages and a final solid: liquid ratio of 1:12. The error bars indicate the standard deviation from three independent experiments.

after 10 h of hydrolysis, which was about 2, 5 and 12-fold of that from A-CS, IL-CS and U-CS, respectively. The lowest lignin content in A-IL-CS (Fig. 1 and Table 1) may be favorable for its enzymatic hydrolysis, and therefore resulted in the highest glucose production with a glucose yield of over 80%. Studies indicate that IL can directly affect enzymatic hydrolysis by reducing crystallinity of CS (Mood et al., 2014). Here, glucose concentration of A-CS hydrolysate was 6 times of that of U-CS; while glucose released from IL-CS was increased by 125% than that of U-CS. Our results suggest that lignin removal was more notable in promoting enzymatic saccharification. Consequently, the enzymatic hydrolysis of A-IL-CS was further optimized.

In order to obtain higher glucose concentration for butanol fermentation, the final solid to liquid ratio was adjusted from 1:15 to 1:12, and the enzyme dosage was optimized to establish an economic saccharification process. As shown in Fig. 3, along with the increase in enzyme dosage from 10 to 70 FPU $g^{-1}_{total\text{-}solid}$, the glucose concentration was also enhanced from 34.0 to 48.9 g L^{-1} after 12 h of hydrolysis. Compared with 70 FPU $g^{-1}_{total\text{-}solid}$, enzyme dosage of 50 FPU $g^{-1}_{total\text{-}solid}$ resulted in nearly similar glucose concentration (48.3 g L^{-1}) after hydrolysis for 10–12 h, and a glucose yield of over 93% was achieved (Fig. 3 and Table 1). Since glucose concentration of about 50 g L^{-1} is required for butanol fermentation, 50 FPU $g^{-1}_{total\text{-}solid}$ was sufficient to achieve desirable glucose concentration.

3.4. Effect of RIL pretreatment on A-CS

One essential requirement for industrial application of ILs is that they must be recyclable for repeated use. In this work, [Bmim][Cl] was recycled after separation of water from the IL by evaporation without removing any soluble fraction in IL. The effectiveness of recycle ionic liquid (RIL) was examined in the pretreatment of A-CS (A-RIL-CS) by measuring the glucose released. [Bmim][Cl] was recycled for ten times as illustrated in Fig. 4. When pretreated by fresh IL, A-CS turned into a gel-like form, mainly because proteins and lipids were dissolved, additionally, part of cellulose, hemicellulose as well as lignin were also dissolved. However, no gel was formed when RIL was used to pretreat A-CS, likely due to the impurities and small amount of water remained in the RIL. Higher glucose level was achieved by fresh IL than that by RIL, indicating that fresh IL could boost the enzymatic hydrolysis by dissolving more cellulose, hemicellulose and lignin. The glucose concentration from A-CS treated by the 10th recycled RIL was 18.7 g L^{-1} after 12 h of hydrolysis, which was 5.5 g L^{-1} lower than that treated by fresh IL.

3.5. Butanol fermentation

The CS hydrolysates pretreated with fresh IL (Hydrolysate I) and IL recycled for ten times (Hydrolysate II) were applied in butanol fermentation by *C. saccharobutylicum* DSM 13864. Control experiments were also carried out using glucose as substrate. The glucose concentrations in controls (Glucose I, 54 g L^{-1}; Glucose II, 50 g L^{-1}) were set the same as the total sugar concentration (determined by DNS) in Hydrolysate I and II. The fermentation results were summarized in Table 2, and the fermentation processes were illustrated in Figs. 5 and 6.

In Glucose I, after about 12 h of lag phase, *C. saccharobutylicum* entered a fast growing stage (Fig. 5(A)). 39.5 g L^{-1} glucose was consumed and butanol titer reached 8.7 g L^{-1} after 65.5 h fermentation with a butanol yield of 0.22 g $g^{-1}_{total\text{-}sugar}$ and productivity of 0.13 g L^{-1} h^{-1}. In fermentation with Hydrolysate I, the lag phase was about 6 h longer than that of Glucose I, and butanol titer

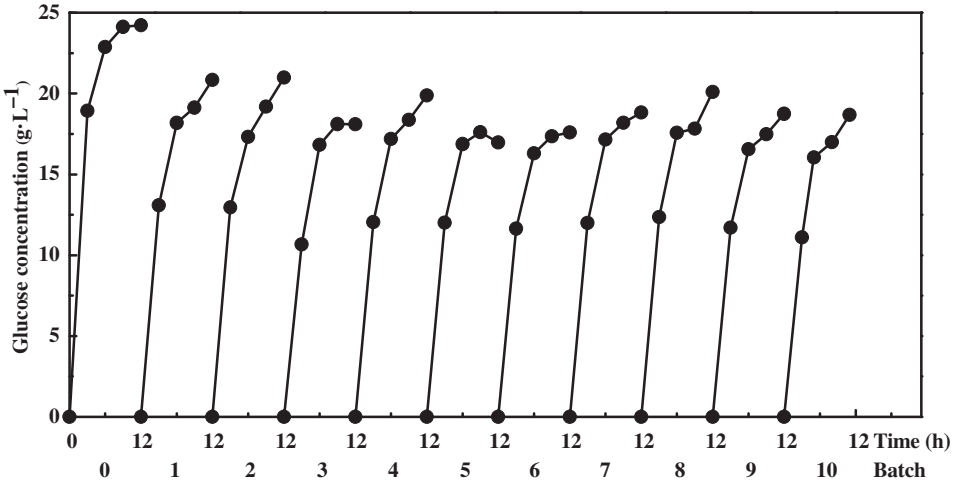


Fig. 4. Enzymatic saccharification of A-CS pretreated by recycled [Bmim][Cl]. 0: IL; 1: 1st RIL; 2: 2nd RIL; 3: 3rd RIL; 4: 4th RIL; 5: 5th RIL; 6: 6th RIL; 7: 7th RIL; 8: 8th RIL; 9: 9th RIL; 10: 10th RIL. A-CS: corn stover that pretreated by alkali for 1 day. The glucose concentration was obtained at a cellulase dosage of 50 FPU $g^{-1}_{total\text{-}solid}$ and a final solid: liquid ratio of 1:20.

Table 2
Butanol fermentation by *C. saccharobutylicum* DSM 13864 using glucose and CS hydrolysate.

Substrate	Total sugar		Butanol			ABE		
	Initial ($g\ L^{-1}$)	Residual ($g\ L^{-1}$)	Titer ($g\ L^{-1}$)	Yield ($g\ g^{-1}_{total\text{-}sugar}$)	Prod.[a] ($g\ L^{-1}\ h^{-1}$)	Titer ($g\ L^{-1}$)	Yield ($g\ g^{-1}_{total\text{-}sugar}$)	Prod.[a] ($g\ L^{-1}\ h^{-1}$)
Glucose I	45.9	6.4	8.7	0.22	0.13	12.3	0.31	0.19
Hydrolysate I[b]	44.1/33.6[d]/7.4[e]	9.1/3.9[d]/4.8[e]	7.4	0.21	0.11	11.7	0.33	0.18
Glucose II	43.7	0.1	10.1	0.23	0.15	14.2	0.33	0.21
Hydrolysate II[c]	40.1/28.3[d]/8.0[e]	5.9/0.7[d]/3.0[e]	7.9	0.23	0.12	12.0	0.35	0.18

[a] Prod.: productivity.
[b] A-IL-CS hydrolysate.
[c] A-RIL-CS hydrolysate.
[d] Glucose concentration.
[e] Xylose concentration.

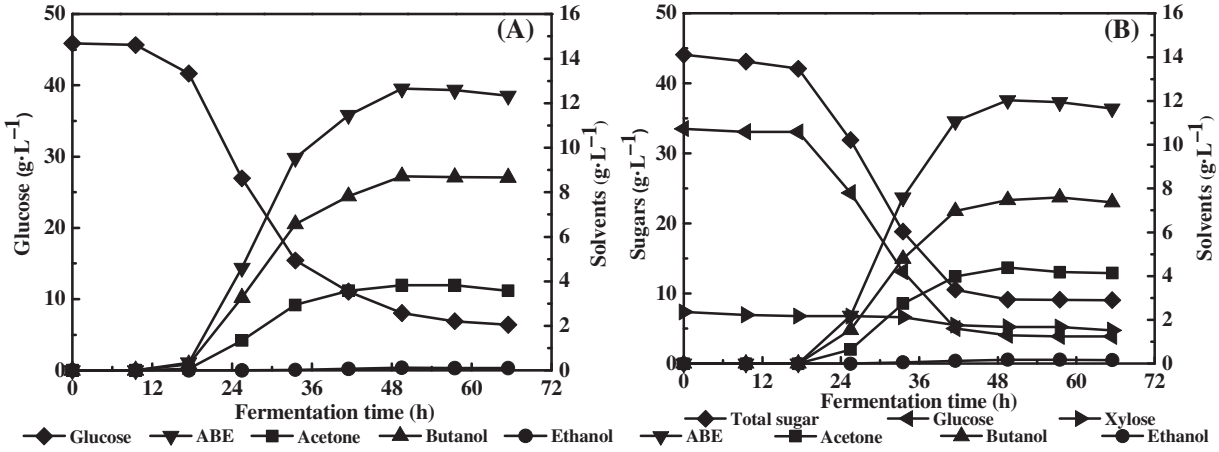


Fig. 5. Butanol fermentation by *C. saccharobutylicum* DSM 13864 using glucose and CS hydrolysate I. (A) Glucose I; (B) Hydrolysate I (A-IL-CS). The fermentation medium and conditions were as follows. (A) and (B) 54 $g\ L^{-1}$ total sugar; 10 $g\ L^{-1}$ corn starch powder; 4 $g\ L^{-1}$ $CaCO_3$; 2 $g\ L^{-1}$ $(NH_4)_2SO_4$; 0.5 $g\ L^{-1}$ K_2HPO_4; 0.01 $g\ L^{-1}$ $MnSO_4{\cdot}H_2O$, 150 mL, pH 6.5, 37 °C, inoculum size 8% (v/v).

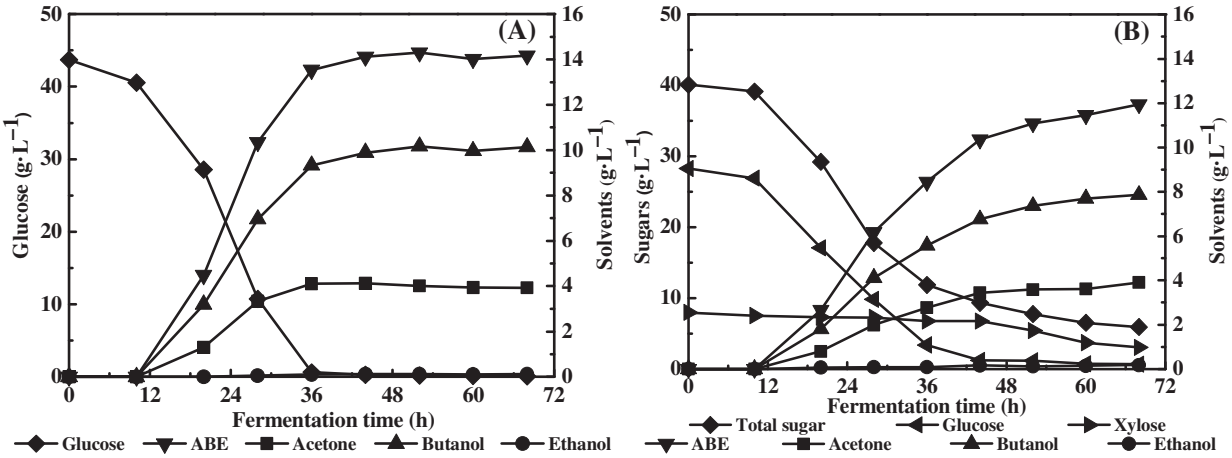


Fig. 6. Butanol fermentation by *C. saccharobutylicum* DSM 13864 using glucose and CS hydrolysate II. (A) Glucose II; (B) Hydrolysate II (A-RIL-CS). The fermentation medium and conditions were as follows. (A) and (B) 50 $g\ L^{-1}$ total sugar; 10 $g\ L^{-1}$ corn starch powder; 4 $g\ L^{-1}$ $CaCO_3$; 2 $g\ L^{-1}$ $(NH_4)_2SO_4$; 0.5 $g\ L^{-1}$ K_2HPO_4; 0.01 $g\ L^{-1}$ $MnSO_4{\cdot}H_2O$, 50 mL, pH 6.5, 37 °C, inoculum size 20% (v/v).

reached the highest level at 50th h when glucose and total sugar dropped to the lowest levels (Fig. 5(B)). Total sugar of 35 $g\ L^{-1}$ was consumed to produce 7.4 $g\ L^{-1}$ butanol with a butanol yield of 0.21 $g\ g^{-1}_{total\text{-}sugar}$ and productivity of 0.11 $g\ L^{-1}\ h^{-1}$. The results were similar to the fermentation of Glucose I, indicating no inhibition effect in Hydrolysate I.

To reduce the cost, [Bmim][Cl] was recycled for up to ten times. Hydrolysate II pretreated by ten times recycled [Bmim][Cl] was used in butanol fermentation, and the control (Glucose II) was also carried out (Fig. 6). For Glucose II, 43.6 $g\ L^{-1}$ glucose was consumed to produce 10.1 $g\ L^{-1}$ butanol after 68 h of fermentation. The butanol yield and productivity were 0.23 $g\ g^{-1}_{total\text{-}sugar}$ and 0.15 $g\ L^{-1}\ h^{-1}$,

respectively. For Hydrolysate II, 7.9 g L^{-1} butanol was obtained, as well as a similar butanol yield (0.23 g $g^{-1}_{total\text{-}sugar}$) and productivity (0.12 g·L^{-1}·h^{-1}).

For Hydrolysate I and II (Table 2), the titers of butanol in fermentation of Hydrolysate II were slightly higher than those in Hydrolysate I, presumably due to a larger inoculum size in Hydrolysate II (20%) than that in Hydrolysate I (8%), as well as less solvents volatilization in a smaller fermentation reactor of Hydrolysate II (100-mL) than that of Hydrolysate I (250-mL). Our results suggest that hydrolysates prepared by both fresh and recycled IL could be used in butanol fermentation without obvious inhibition. No significant difference between hydrolysates prepared by fresh IL and recycled IL was observed. It was also noticed that *C. saccharobutylicum* could utilize xylose in the fermentation process, the xylose consumption in Hydrolysate I and II was 2.6 and 5.0 g L^{-1}, respectively.

4. Conclusion

Corn stover was pretreated using dilute NaOH (1 wt%) followed by recycled IL. The glucose released from A-IL-CS achieved up to 32.9 g L^{-1}, which was increased by 90%, 430% and 1,094% compared with A-CS, IL-CS and U-CS, respectively. [Bmim][Cl] recycled for ten times was applied in the pretreatment of A-CS without obvious loss of efficiency. The A-IL-CS and A-RIL-CS hydrolysates were successfully applied in butanol fermentation by *C. saccharobutylicum* without inhibitory effect. The yield and productivity of butanol reached 0.23 g $g^{-1}_{total\text{-}sugar}$ and 0.12 g L^{-1} h^{-1} after 68 h of fermentation.

Acknowledgements

We are grateful to National Natural Science Foundation of China (21276112, 31401634), Natural Science Foundation of Jiangsu Province (BK20150003), the Fundamental Research Funds for the Central Universities (JUSRP51409B), the Program of Introducing Talents of Discipline to Universities (111-2-06), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions for the financial support of this research.

References

Baral, N.R., Li, J., Jha, A.K., 2014. Perspective and prospective of pretreatment of corn straw for butanol production. Appl. Biochem. Biotechnol. 172 (2), 840–853.
Behera, S., Arora, R., Nandhagopal, N., Kumar, S., 2014. Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. Renew. Sustain. Energy Rev. 36, 91–106.
Cetinkol, O.P., Dibble, D.C., Cheng, G., Kent, M.S., Knierim, B., Auer, M., Wemmer, D. E., Pelton, J.G., Melnichenko, Y.B., Ralph, J., Simmons, B.A., Holmes, B.M., 2010. Understanding the impact of ionic liquid pretreatment on eucalyptus. Biofuels 1, 33–46.
Chum, H.L., Johnson, D.K., Black, S., Baker, J., Grohmann, K., Sarkanen, K.V., Wallace, K., Schroeder, H.A., 1988. Organosolv pretreatment for enzymatic hydrolysis of poplars: I. Enzyme hydrolysis of cellulosic residues. Biotechnol. Bioeng. 31, 643–649.
Demirbas, A., 2008. Biofuels sources, biofuel policy, biofuel economy and global biofuel projections. Energy Convers. Manage. 49 (8), 2106–2116.
Ezeji, T., Blaschek, H.P., 2008. Fermentation of dried distillers' grains and solubles (DDGS) hydrolysates to solvents and value-added products by solventogenic clostridia. Bioresour. Technol. 99 (12), 5232–5242.
Galbe, M., Zacchi, G., 2007. Pretreatment of lignocellulosic materials for efficient bioethanol production. Adv. Biochem. Eng. Biotechnol. 108, 41–65.
Geng, X., Henderson, W.A., 2012. Pretreatment of corn stover by combining ionic liquid dissolution with alkali extraction. Biotechnol. Bioeng. 109 (1), 84–91.
Kim, T.H., Kim, J.S., Sunwoo, C., Lee, Y.Y., 2003. Pretreatment of corn stover by aqueous ammonia. Bioresour. Technol. 90 (1), 39–47.
Kosan, B., Michels, C., Meister, F., 2007. Dissolution and forming of cellulose with ionic liquids. Cellulose 15 (1), 59–66.
Lee, S.H., Doherty, T.V., Linhardt, R.J., Dordick, J.S., 2009. Ionic liquid-mediated selective extraction of lignin from wood leading to enhanced enzymatic cellulose hydrolysis. Biotechnol. Bioeng. 102 (5), 1368–1376.
Lee, S.Y., Park, J.H., Jang, S.H., Nielsen, L.K., Kim, J., Jung, K.S., 2008. Fermentative butanol production by *Clostridia*. Biotechnol. Bioeng. 101 (2), 209–228.
Li, C., Knierim, B., Manisseri, C., Arora, R., Scheller, H.V., Auer, M., Vogel, K.P., Simmons, B.A., Singh, S., 2010. Comparison of dilute acid and ionic liquid pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. Bioresour. Technol. 101 (13), 4900–4906.
Li, L., Ai, H., Zhang, S., Li, S., Liang, Z., Wu, Z.Q., Yang, S.T., Wang, J.F., 2013. Enhanced butanol production by coculture of *Clostridium beijerinckii* and *Clostridium tyrobutyricum*. Bioresour. Technol. 143, 397–404.
Lloyd, A., 1984. French push ahead with biomass conversion. New Sci. 102, 21.
MacDonald, D.G., Bakhshi, N.N., Mathews, J.F., Roychowdhury, A., Bajpai, P., Moo-Young, M., 1983. Alkali treatment of corn stover to improve sugar production by enzymatic hydrolysis. Biotechnol. Bioeng. 25, 2067–2076.
Mood, S.H., Golfeshan, A.H., Tabatabaei, M., Abbasalizadeh, S., Ardjmand, M., Jouzani, G.S., 2014. Comparison of different ionic liquids pretreatment for corn stover enzymatic saccharification. Prep. Biochem. Biotechnol. 44 (5), 451–463.
Nasirpour, N., Mousavi, S.M., Shojaosadati, S.A., 2014. A novel surfactant-assisted ionic liquid pretreatment of sugarcane bagasse for enhanced enzymatic hydrolysis. Bioresour. Technol. 169, 33–37.
Ni, Y., Sun, Z., 2009. Recent progress on industrial fermentative production of acetone–butanol–ethanol by *Clostridium acetobutylicum* in China. Appl. Microbiol. Biotechnol. 83 (3), 415–423.
Ni, Y., Wang, Y., Sun, Z., 2012. Butanol production from cane molasses by *Clostridium saccharobutylicum* DSM 13864: batch and semicontinuous fermentation. Appl. Biochem. Biotechnol. 166 (8), 1896–1907.
Ni, Y., Xia, Z., Wang, Y., Sun, Z., 2013. Continuous butanol fermentation from inexpensive sugar-based feedstocks by *Clostridium saccharobutylicum* DSM 13864. Bioresour. Technol. 129, 680–685.
Ouajai, S., Shanks, R.A., 2005. Composition, structure and thermal degradation of hempcellulose after chemical treatments. Polym. Degrad. Stab. 89 (2), 327–335.
Qureshi, N., Ezeji, T.C., Ebener, J., Dien, B.S., Cotta, M.A., Blaschek, H.P., 2008. Butanol production by *Clostridium beijerinckii*. Part I: use of acid and enzyme hydrolyzed corn fiber. Bioresour. Technol. 99 (13), 5915–5922.
Qureshi, N., Lolas, A., Biaschek, H.P., 2001. Soy molasses as fermentation substrate for production of butanol using *Clostridium beijerinckii* BA101. J. Ind. Microbiol. Biotechnol. 26, 290–295.
Qureshi, N., Saha, B.C., Cotta, M.A., 2007. Butanol production from wheat straw hydrolysate using *Clostridium beijerinckii*. Bioprocess Biosyst. Eng. 30 (6), 419–427.
Qureshi, N., Saha, B.C., Dien, B., Hector, R.E., Cotta, M.A., 2010a. Production of butanol (a biofuel) from agricultural residues: Part I – use of barley straw hydrolysate. Biomass Bioenergy 34 (4), 559–565.
Qureshi, N., Saha, B.C., Hector, R.E., Dien, B., Hughes, S., Liu, S., Iten, L., Bowman, M.J., Sarath, G., Cotta, M.A., 2010b. Production of butanol (a biofuel) from agricultural residues: Part II – use of corn stover and switchgrass hydrolysates. Biomass Bioenergy 34 (4), 566–571.
Saha, B.C., Iten, L.B., Cotta, M.A., Wu, Y.V., 2005. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochem. 40 (12), 3693–3700.
Samayam, I.P., Schall, C.A., 2010. Saccharification of ionic liquid pretreated biomass with commercial enzyme mixtures. Bioresour. Technol. 101 (10), 3561–3566.
Sathitsuksanoh, N., Zhu, Z., Zhang, Y.H.P., 2012. Cellulose solvent-based pretreatment for corn stover and avicel: concentrated phosphoric acid versus ionic liquid [BMIM]Cl. Cellulose 19 (4), 1161–1172.
Schell, D.J., Walter, P.J., Johnson, D.K., 1992. Dilute sulfuric acid pretreatment of corn stover at high solids concentrations. Appl. Biochem. Biotechnol. 34–35, 659–665.
Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluitter, J., Templeton, D., Crocker, D., 2008. Determination of structural carbohydrates and lignin in biomass. Laboratory Analytical Procedure. National Renewable Energy Laboratory, Golden, Colorado, 29.
Yang, B., Wyman, C.E., 2004. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. Biotechnol. Bioeng. 86 (1), 88–95.
Zavrel, M., Bross, D., Funke, M., Buchs, J., Spiess, A.C., 2009. High-throughput screening for ionic liquids dissolving lignocellulose. Bioresour. Technol. 100 (9), 2580–2587.
Zhang, T., Du, N., Tan, T., 2011. Biobutanol production from sweet sorghum bagasse. J. Biobased Mater. Bioenergy 5 (3), 331–336.