Short Communication

Enhancing cellulose accessibility of corn stover by deep eutectic solvent pretreatment for butanol fermentation

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HIGHLIGHTS

- Seven deep eutectic solvents were prepared and applied in corn stover (CS) pretreatment.
- ChCl:formic acid showed best performance with $17.0 \, g \, L^{-1}$ glucose in CS hydrolysate.
- CS hydrolysate pretreated by ChCl:formic acid was successfully applied in butanol fermentation.

GRAPHICAL ABSTRACT
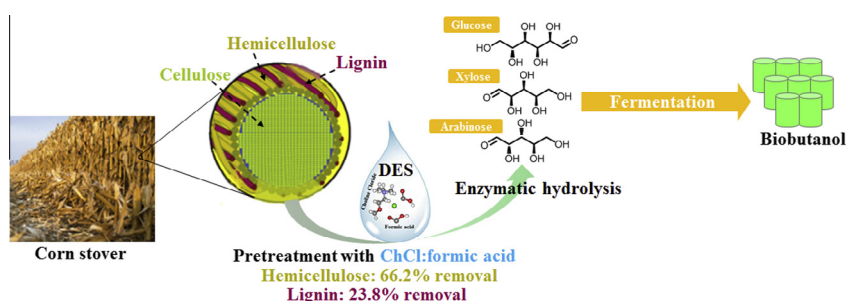
ABSTRACT

In this study, an effective corn stover (CS) pretreatment method was developed for biobutanol fermentation. Deep eutectic solvents (DESs), consisted of quaternary ammonium salts and hydrogen donors, display similar properties to room temperature ionic liquid. Seven DESs with different hydrogen donors were facilely synthesized. Choline chloride:formic acid (ChCl:formic acid), an acidic DES, displayed excellent performance in the pretreatment of corn stover by removal of hemicellulose and lignin as confirmed by SEM, FTIR and XRD analysis. After optimization, glucose released from pretreated CS reached $17.0 \, g \, L^{-1}$ and yield of 99%. The CS hydrolysate was successfully utilized in butanol fermentation by *Clostridium saccharobutylicum* DSM 13864, achieving butanol titer of $5.63 \, g \, L^{-1}$ with a yield of $0.17 \, g \, g^{-1}$ total sugar and productivity of $0.12 \, g \, L^{-1} \, h^{-1}$. This study demonstrates DES could be used as a promising and biocompatible pretreatment method for the conversion of lignocellulosic biomass into biofuel.

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1. Introduction

Biobutanol is a promising biofuel that has the potential to mitigate the issues of diminishing oil resources, global warming, and environmental pollution, due to its higher energy content, a lower hydroscopicity and volatility, a better blending ability with gasoline (Ni et al., 2012). Recently, great efforts have been committed in the production of biobutanol from renewable lignocellulosic resources, mainly from agricultural residues, forestry sources,

industrial residues, municipal solid wastes and energy crops, using *Clostridium* which could utilize both hexoses and pentoses (Ni and Sun, 2009). Lignocellulosic feedstocks, such as corn stover (CS) mainly composed of cellulose (40–60%), hemicellulose (20–40%) and lignin (10–24%), provide low-cost and abundant resource for biobutanol production (Geng and Henderson, 2012).

The recalcitrant structure and poor solubility of cellulose in water have made cellulose hydrolysis in high yield quite challenging (Meng and Ragauskas, 2014). To achieve efficient enzymatic conversion of CS into fermentable sugars, development of effective pretreatment strategies to increase the accessibility of cellulose is essential. An efficient, economic and green pretreatment method

should satisfy the following conditions: cost-effective, none or less inhibitors, enhanced enzymatic hydrolysis, easy operation and recycle, and independent of special equipment (George et al., 2015). Numerous pretreatment strategies (e.g., physical, chemical, physicochemical methods etc.) have been developed, and the advantages and disadvantages have been well-reviewed recently (Mora-Pale et al., 2011; Meng and Ragauskas, 2014). Ionic liquids are regarded to be promising and efficient in regenerating nearly complete amorphous cellulose and disrupting inter- and intro-molecular hydrogen bonds among cellulose, hemicellulose and lignin (Brandt et al., 2013). Moreover, ILs are environmentally benign and compatible with organisms, independent on biomass types and can be recycled and reused with little loss (He et al., 2015a). However, the general high-cost for high purity ILs has raised concerns for scale-up application. Consequently, the design of low-cost ionic liquids is attracting increasing attention.

Various ionic liquids composed of different cations and hydrogen donors have been designed to increase the accessible binding sites of cellulose for cellulose (George et al., 2015). Deep eutectic solvents (DESs) derived from natural and renewable components were first synthesized by Abbott in 2004 and could be formed between a variety of quaternary ammonium salts and hydrogen donors (Abbott et al., 2004). The physical properties of DES, such as low viscosity, melting points and volatility, high thermal stability, conductivity and surface tension, are similar to ambient temperature ionic liquids. The ease of synthesis at low cost (about 20% to traditional ILs), availability and biodegradability make DESs versatile alternatives to ionic liquids (Gorke et al., 2008). Gunny and coworkers evaluated the application of three deep eutectic solvents, made up of choline chloride and glycerol (ChCl:glycerol), ethylene glycol (ChCl:ethylene glycol) and malonic acid (ChCl:malonic acid), in the pretreatment of rice husk, and higher glucose yield and lower energy consumption were achieved when compared with diluted alkali system (Gunny et al., 2015). Choline chloride imidazole (ChCl:imidazole) was proved to be effective in the pretreatment of corncob by reducing lignin and hemicellulose content, producing 76% fermentable sugars of the theoretical carbohydrates.

It has been reported that acidic ILs with HSO_4^- could act as good pretreatment solvent for the dissolve of lignocellulosic biomass (Li et al., 2008). Some organic acids, including formic acid (Zhang et al., 2010), acetic acid (Trzcinski and Stuckey, 2015) and oxalic acid (Kundu et al., 2015) etc, have recently been introduced as pretreatment reagents to break the recalcitrant structure of lignocellulosic materials by similar mechanism as diluted acids. We thus speculate that deep eutectic solvents with acidic hydrogen donors might be superior to ILs in increasing the cellulose accessibility of lignocellulosic materials. Five acidic and two neutral deep eutectic solvents were synthesized and evaluated in the pretreatment of CS. The efficacy of pretreatment was compared with that of commercial ionic liquid ([Bmim][Cl]). Finally, by combining pretreatment with enzymatic hydrolysis, the hydrolysate was utilized in butanol fermentation.

2. Methods

2.1. Strains, biomass, and chemicals

Corn stover (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. 1-Butyl-3-methylimidazolium chloride ([Bmim][Cl], 99%) was purchased from Henan Lihua Pharmaceutical Co., Ltd. Choline chloride (ChCl, ⩾98%) was obtained from Shanghai Titanchem Co., Ltd. ACCELLERASE® 1500 cellulase was a generous gift from

Genencor (Wuxi, China) Bio-Products Co. All other chemicals were reagent or analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. Clostridium saccharobutylicum DSM 13864 was stored and cultured as our previously reported (Ding et al., 2016).

2.2. Synthesis of choline chloride based DESs

Chloline chloride as ammonium salt and seven hydrogen bond donor molecules were mixed in the ratio as listed in Table S1. The mixture was heated and shook at 180 rpm, 30 or 60 °C in a conical flask with plug to reduce volatilization until a homogenous colorless liquid was formed as introduced by Abbott (Abbott et al., 2004). After that, the synthesized DESs were heated at around 80 °C for 12 h to remove the unreacted free acid. The DESs were kept in a vacuum desiccator with silica gel for further use.

2.3. General protocol for pretreatment of CS with DESs and [Bmim][Cl]

Seven DESs and [Bmim][Cl] (100 g) were added into a 1 L three-necked flask and heated to 130 °C. Then 5.0 g untreated corn stover (U-CS) was supplemented and incubated at 130 °C for 2 h with mechanical agitation (100 rpm). About 0.6 L hot deionized water (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 several times with hot deionized water (85 °C) to remove the residual DESs or [Bmim][Cl]. The pretreated CS was dried at 60 °C and stocked in sealed plastic bags. The CS pretreated by DESs or [Bmim][Cl] was defined as X-CS (X refers to the seven DESs or [Bmim][Cl]).

2.4. General protocol for enzymatic hydrolysis of pretreated CS

The pretreated CS (1.50 g) was added into a 50 mL conical flask with plug, and mixed with 30 mL of citrate buffer (50 mM, pH 4.8), ampicillin (100 μL at 1 g L^{-1}) and cellulase (50 FPU g^{-1} total-solid). The mixture was incubated in a water bath at 50 °C and 120 rpm for 72 h. Samples (300 μL) were withdrawn at 6, 12, 24, 48, 72 h, and centrifuged at 12,000 g for 10 min. The resultant supernatants (100 μL) were mixed with 900 μL diluted H_2SO_4 (0.4% w/w) to terminate the hydrolysis reaction. The glucose concentration was determined as previously described (Ding et al., 2016).

2.5. Analytical methods and formulas

The amount of cellulose, hemicellulose, lignin as well as ash in the in pretreated CS (U-CS, [Bmim][Cl]-CS and ChCl:formic acid-CS) were analyzed we previously reported (Ding et al., 2016). The morphology of the pretreated CS was observed with scanning electron microscopy (5.0 kV, ×1200 Hitachi S-4800, Japan). Chemical structure of the pretreated CS was analyzed with Fourier Transform Infra-Red (FTIR, 400–4000 cm^{-1}, Nicolet IS10). Crystallinity of the pretreated CS was measured by the X-Ray diffractometer (XDR) using the Cu kα radiation source at 40 kV and 40 mA from 8° to 40° with a scanning speed of 2°/min (D8 Advance XRD, Bruker, Germany). The crystallinity index (CrI), glucose yield, removal rate of hemicellulose and lignin were calculated according to a previously reported method (Segal et al., 1959; Chen et al., 2009)

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100\% \tag{1}$$

I_{002} and I_{am} are the intensity of the peaks at 2θ of near 22.0° and 17.8° respectively.

$$\text{Yield of glucose}(\%) = \frac{[\text{Glucose released}]}{[\text{Cellulose in U-CS}] \times 1.11} \times 100\% \tag{2}$$

Removal of hemicellulose(%)

$$= \left(1 - \frac{[\text{Hemicellulose in pretreated-CS}]}{[\text{Hemicellulose in U-CS}]} \times \text{solid yield} \right) \times 100\% \quad (3)$$

Removal of lignin(%) $= \left(1 - \frac{[\text{Lignin in pretreated-CS}]}{[\text{Lignin in U-CS}]} \times \text{solid yield} \right)$

$$\times 100\% \quad (4)$$

2.6. Optimization of CS pretreatment with ChCl:formic acid

Effects of temperature and time on the pretreatment of CS with ChCl:formic acid were performed using the general pretreatment protocol except for the temperatures of 90, 110 and 130 °C, pretreatment time of 0.5, 1.0, 2.0 and 3.0 h. The ratio of CS and ChCl:formic acid on the pretreatment was optimized by mixing of 5.0, 6.7 and 10.0 g CS with 100 g ChCl:formic acid to achieve the ratio of 1:20, 1:15 and 1:10 employing the general pretreatment protocol. Afterwards the amount of glucose released from pretreated CS was measured according to general enzymatic hydrolysis protocol. All the experiments were carried out in triplicates.

Different amount of CS (2.00, 1.33, 1.00 g) pretreated by ChCl:formic acid were added into 50 mL conical flasks with plugs, and then mixed with 20 mL of citrate buffer (50 mM, pH 4.8), ampicillin (100 μL, 1 g L^{-1}) and 50 FPU cellulase at pretreated CS to enzyme dosage ratio of 40, 26.6 and 20 mg$_{CS}$·FPU^{-1}. Afterwards the pretreated CS was hydrolyzed and the glucose concentration was determined using the general enzymatic hydrolysis protocol. All the experiments were carried out in triplicates.

2.7. Biobutanol fermentation using CS hydrolysates by C. saccharobutylicum DSM 13864

The CS hydrolysates (150 mL) were prepared using the optimized conditions and concentrated as previously described (Ding et al., 2016), and evaluated as medium for biobutanol fermentation by mixing with other components (gram per liter): corn starch powder, 10; CaCO$_3$, 4; (NH$_4$)$_2$SO$_4$, 2; K$_2$HPO$_4$, 0.5; MnSO$_4$·H$_2$O, 0.01. Control experiment was carried out using the same medium except for with glucose as carbon source. The pH of the medium was adjusted to 6.5 with NaOH and sterilized at 115 °C for 20 min. Actively growing culture (10% v/v) was inoculated in the mediums and anaerobically fermented at 37 °C in a desiccator (0.065 MPa) (Ding et al., 2016). After inoculation, the initial total sugars of control and CS hydrolysate were determined to be 51.7 and 48.2 g L^{-1} respectively. The ABE and acids contents were quantified by GC analysis according to previous report (Ni et al., 2013). The fermentation was carried out in triplicates.

3. Results and discussion

3.1. Screening of deep eutectic solvents for CS pretreatment

According to previous report (Abbott et al., 2004), seven deep eutectic solvents (DESs) were facilely synthesized by mixing choline chloride and hydrogen donors, including urea, glycerol, formic acid, acetic acid, oxalic acid, malonic acid and citric acid to form ChCl:urea, ChCl:glycerol, ChCl:formic acid, ChCl:acetic acid, ChCl:oxalic acid, ChCl:malonic acid and ChCl:citric acid. Under the same pretreated condition at 130 °C for 2 h, the effects of these seven DESs on CS pretreatment were compared with commercial [Bmimi][Cl], which showed good performance in increasing cellulose accessibility (Mora-Pale et al., 2011). The glucose concentration after cellulose hydrolysis were determined and illustrated in Fig. 1. The highest glucose concentration of 15.7 g L^{-1} was obtained using ChCl:formic acid, representing a theoretical glucose yield of

91.5%. Unlike other pretreatments, the glucose released from ChCl:formic acid pretreated-CS showed a steady increase during 72 h of enzymatic hydrolysis, indicating its high efficiency in enhancing the cellulose accessibility and biocompatibility to cellulase activity. Pretreatment with [Bmim][Cl] resulted in about 6.1 g L^{-1} glucose, a little lower than that of ChCl:acetic acid, ChCl:oxalic acid and ChCl:malonic acid. No obvious effect was found with ChCl:citric acid, ChCl:urea and ChCl:glycerol on the pretreatment of CS. Gunny and coworkers have reported the effectiveness of ChCl:urea and ChCl:glycerol in pretreatment of rice husk in 10 mL reaction system, the difference might ascribe to the different lignocellulosic biomass and operation procedure (Gunny et al., 2015).

According to Fig. 1, acidic DESs exhibited good performance in CS pretreatment. Moreover, as the number of carboxylic group and chain length increased, the glucose concentration decreased, especially for ChCl:citric acid. No apparent pattern is observed with the increase in acidity of hydrogen donors, since the acidic sequence is citric acid > malonic acid > oxalic acid > formic acid > acetic acid. The specific mechanism of ChCl:formic acid in CS pretreatment needs further investigation.

3.2. Chemical compositions of U-CS, ChCl:formic acid-CS and [Bmim][Cl]-CS

The chemical composition of U-CS, ChCl:formic acid-CS and [Bmim][Cl]-CS are summarized in Table 1. The solid yields of ChCl:formic acid-CS and [Bmim][Cl]-CS are 69.4% and 93.0% respectively. Cellulose content increased from 31.0% to 47.9% after pretreatment employing ChCl:formic acid, 12.1% higher than that of [Bmim][Cl], owing to the enhanced cellulose accessibility in saccharification. It is remarkable that ChCl:formic acid is highly efficient in the removal of hemicellulose, with only 9.8% hemicellulose residual in pretreated CS and a removal rate of 66.2%. However, little effect was found with [Bmim][Cl] in the disruption of hemicellulose as reported (Mood et al., 2014). It was noticed that the absolute lignin content in ChCl:formic acid-CS (27.1%) is slightly higher than that of [Bmim][Cl]-CS (23.9%) and U-CS (24.1%). Besides that, 23.8% lignin was removed from CS by ChCl:formic acid, higher than 8.5% of [Bmim][Cl]. In the calculation of the relative removal rate of lignin and hemicellulose, solid yield was taken into account as shown in formulas (3) and (4). Therefore, the high lignin removal rate in ChCl:formic acid pretreated CS is ascribed to the low solid yield (69.4%) and high efficiency in breaking the recalcitrance of CS, while the lower lignin removal rate of
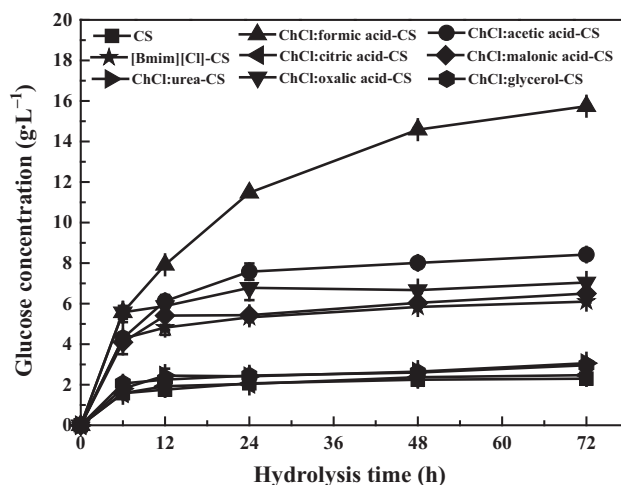

Fig. 1. Enzymatic hydrolysis of the pretreated CS by seven DESs and [Bmim][Cl].

Table 1
Chemical composition and CrI of corn stover pretreated with [Bmim][Cl] and ChCl:formic acid.

	U-CS	[Bmim][Cl]-CS	ChCl:formic acid-CS
Composition/%			
Cellulose	31.0 ± 1.7	35.8 ± 0.3	47.9 ± 0.3
Hemicellulose	20.1 ± 0.8	21.6 ± 0.4	9.8 ± 1.6
Acid-insoluble lignin	24.1 ± 0.6	23.9 ± 0.9	27.1 ± 1.2
Acid-soluble lignin	1.3 ± 0.0	1.1 ± 0.0	0.8 ± 0.0
Ash	8.6 ± 0.6	4.6 ± 0.4	7.1 ± 0.6
Solid yield	–	93.0	69.4
Hemicellulose removal	–	0.1	66.2
Lignin removal	–	8.5	23.8
CrI	31.1	41.1	57.2

[Bmim][Cl] is assumed to be caused by regeneration. Chemical composition analysis indicates ChCl:formic acid could increase the cellulose accessibility by reducing the hemicellulose and lignin.

3.3. Properties of U-CS, ChCl:formic acid-CS and [Bmim][Cl]-CS

SEM analysis (Figs. S1–S3) shows the morphology and physical changes of U-CS, ChCl:fomic acid-CS and [Bmim][Cl]-CS. A stiff bundle of small fibers with flat surface and porous internal is observed in U-CS. No apparent difference is found in [Bmim][Cl]-CS which is in agreement with previous report (Mood et al., 2014). In the ChCl: formic acid-CS, the surface became loose with some small particles attached, which might be the residual hemicellulose and lignin aggregates. Although ChCl:formic acid-CS is also composed of fibers in bundles, the internally porous structure is absent which

could be caused by the disruption of inter- and intra-hydrogen bonds of cellulose fibrils. The structural difference indicates the removal of hemicellulose and lignin by ChCl:formic acid treatment, which improves cellulose accessibility.

Crystallinity is regarded as an important factor in enzymatic hydrolysis. XRD assay were performed (details in Fig. S4). As shown in Table 1, the crystallinity index (CrI) of U-CS was calculated to be 31.1, because of the high content of non-cellulosic material. A higher CrI of 41.1 was determined for [Bmim][Cl]-CS, likely caused by the incomplete swollen of cellulose fibers prevented by lignin or the recrystallization in regeneration step (Geng and Henderson, 2012). The CrI of ChCl:formic acid-CS is 57.2, ranking the highest among three samples tested. It was reported that the increased CrI after pretreatment is resulted from the removal of non-crystallinity components including hemicellulose and lignin, which leads to the increased proportion of cellulose (He et al., 2015b). In this study, pretreatment with ChCl:formic acid resulted the highest CrI of ChCl:formic acid-CS, indicating the effectiveness of this pretreatment process in enhancing the cellulose accessibility for enzymatic hydrolysis, which is also in agreement with the high glucose concentration as illustrated in Fig. 1.

FTIR analysis was carried out to evaluate the changes of different chemical composition in the pretreated CS (details in Fig. S5). A decrease in absorbance at near 1730 cm^{-1} could be attributed to the carbonyl functional groups in hemicellulose. As shown in Table 1, about 66.2% of hemicellulose has been removed. The band near 1315 cm^{-1} could be caused by the C–H bending vibrations in cellulose and hemicellulose (He et al., 2015a), suggesting the pretreated CS still contains considerable amount of crystalline cellulose. The band near 890 cm^{-1} is related to the C–O–C stretching
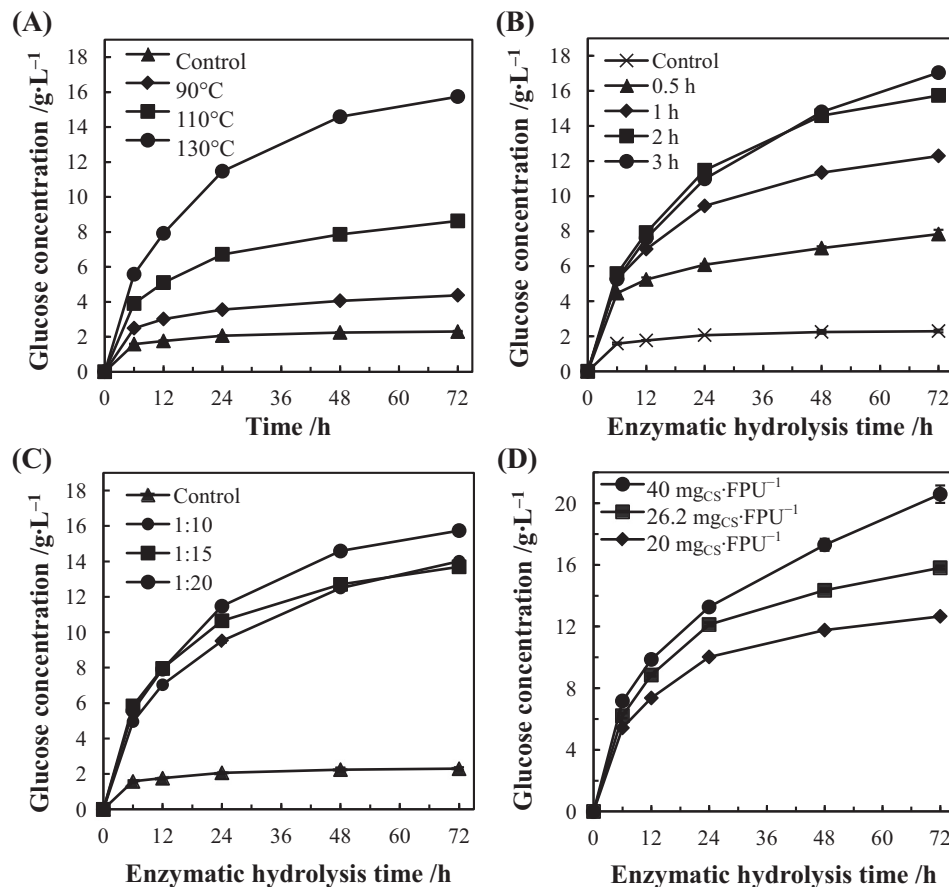

Fig. 2. Optimization of enzymatic hydrolysis of ChCl:formic acid pretreated CS. (A) temperature, (B) time, (C) solid to liquid ratio, (D) pretreated CS to enzyme dosage ratio.
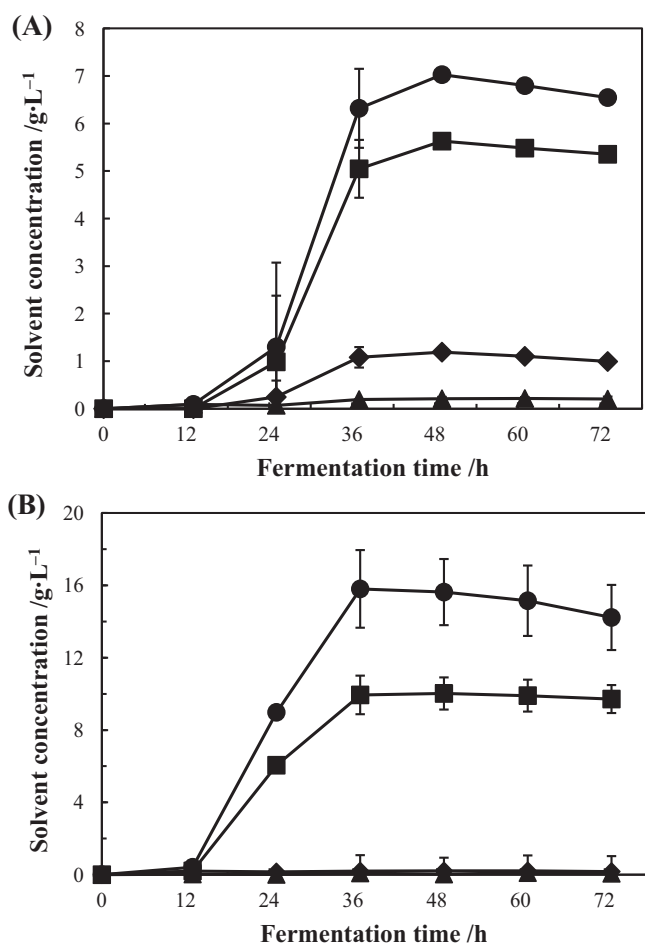
Fig. 3. Butanol fermentation using ChCl:formic acid-CS hydrolysate (A) and glucose as carbon sources (B) by *C. sacchrobutylicum* DSM 13864. (●) total solvent; (■) butanol; (◆) acetone; (▲) ethanol.

at the β-1,4-glycosidic linkage existed in the amorphous cellulose. These results substantiate the structure of the pretreated CS was obviously changed, resulting in enhanced cellulose accessibility for cellulase hydrolysis.

3.4. Optimization of CS pretreatment with ChCl:formic acid

To achieve the maximum utilization of ChCl:formic acid in the pretreatment of CS, the conditions affecting the total solid and glucose yield were optimized, including temperature, time, solid to liquid ratio, and the pretreated CS to enzyme dosage ratio. From Fig. 2(A), the glucose concentration increased from 4.37 g L^{-1} to 15.7 g L^{-1}, as the increase of pretreatment temperature from 90 °C to 130 °C. Considering the higher fermentable sugars in hydrolysate, 130 °C is regarded as the suitable temperature. Incubation time was optimized over 0.5–3 h (Fig. 2(B)). The final glucose concentration increased rapidly from 7.83 g L^{-1} (0.5 h) to 15.7 g L^{-1} (2 h). When pretreatment time was further elongated to 3 h, the glucose concentration and yield reached the highest level, 17.0 g L^{-1} and 99.0% respectively. The solid to liquid ratio was also optimized to obtain the highest fermentable sugars from CS. As shown in Fig. 2(C), glucose concentration is similar at solid: liquid ratio of 1:10 and 1:15. When further increased to 1:20, the glucose concentration reached 15.7 g L^{-1}, corresponding to a theoretical yield of 91.5%. Ratio of pretreated CS to enzyme dosage has significant effect on glucose concentration (Fig. 2(D)). At 20, 26.6 and $40 \text{ mg}_{CS} \text{ FPU}^{-1}$, the glucose concentration and yield were

12.7 g L^{-1} and 73.6%, 15.8 g L^{-1} and 69.1%, 20.6 g L^{-1} and 59.8% respectively. Further increase the pretreated CS to enzyme dosage ratio led to high mass transfer resistance for enzymatic saccharification. As a result, $40 \text{ mg}_{CS} \text{ FPU}^{-1}$ is regarded as the best loading ratio of pretreated CS to enzyme dosage.

3.5. Biobutanol fermentation using ChCl:formic acid-CS hydrolysate

DESs are regarded to be biocompatible for enzyme activity (Lehmann et al., 2014). Although several DESs (e.g., ChCl:glycerol, ChCl:urea and ChCl:imidazole etc.,) have been applied in the pretreatment of lignocellulosic materials (Gunny et al., 2015; Procentese et al., 2015), the application of their corresponding hydrolysates in biofuels production, especially biobutanol, have not been reported. Here, butanol fermentation from ChCl:formic acid-CS hydrolysate was performed using *C. saccharobutylicum* DSM 13864. The time course of solvent production and residual sugars were shown in Fig. 3 and Fig. S6. No obvious inhibition effect is observed. At 48 h, the butanol concentration reached its highest level of 5.63 g L^{-1}, about 34.0 g L^{-1} total sugar was consumed, including 26.1 g L^{-1} glucose, 7.87 g L^{-1} xylose and 0.06 g L^{-1} arabinose. The butanol yield and productivity are 0.17 g g^{-1} total sugar and $0.12 \text{ g L}^{-1} \text{ h}^{-1}$, which is comparable with those of the glucose control (0.21 g g^{-1} total sugar and $0.21 \text{ g L}^{-1} \text{ h}^{-1}$). The yield and productivity of total solvent are 7.03 g g^{-1} total sugar and $0.15 \text{ g L}^{-1} \text{ h}^{-1}$ (details in Table S2). Therefore, ChCl: formic acid was successfully applied in CS pretreatment for biobutanol fermentation.

4. Conclusion

In this study, seven DESs were synthesized and evaluated in the pretreatment of CS. ChCl:formic acid, DES with acidic hydrogen donor, could increase cellulose accessibility by removal of hemicellulolose and lignin, leading to a high CrI of 57.1. In butanol fermentation using DES pretreated hydrolysates by *Clostridium saccharobutylicum* DSM 13864, butanol yield of 0.17 g g^{-1} total sugar and productivity of $0.12 \text{ g L}^{-1} \text{ h}^{-1}$ were attained without apparent inhibitory effect. Taken together, this work demonstrates that DESs pretreatment is a promising and biocompatible procedure in the biofuel production from lignocellulosic biomass.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.11.002.

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