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Novel dihydrogen-bonding deep eutectic solvents: Pretreatment of rice straw for butanol fermentation featuring enzyme recycling and high solvent yield

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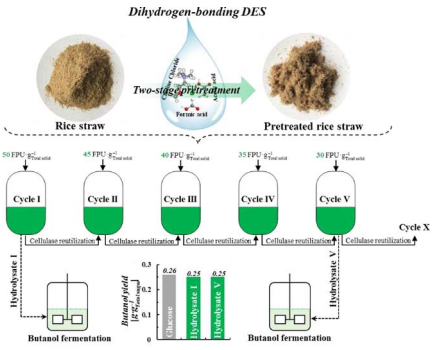
GRAPHICAL ABSTRACT



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ABSTRACT

Rice straw (RS) is one of the most abundant lignocellulosic biomasses in China, which contains mainly of cellulose, hemicellulose and lignin. In this study, a novel deep eutectic solvent (DES), foraceline, was prepared with choline chloride and double hydrogen bond donors (formic acid and acetic acid). By combining foraceline and sodium carbonate (1%), a two-stage pretreatment method was developed and applied in RS pretreatment to improve the biodegradability of lignocellulose. The highest glucose and total sugar of 37.3 $g{\cdot}L^{-1}$ and 42.8 $g{\cdot}L^{-1}$ were obtained after hydrolysis of 24 h using 50 $FPU{\cdot}g^{-1}_{total\ solid}$ of cellulase. The cellulase attached to RS was re-utilized for five continuous cycles in which enzyme loading was reduced from 50 $FPU{\cdot}g^{-1}_{total\ solid}$ (Cycle I) to 30 $FPU{\cdot}g^{-1}_{total\ solid}$ (Cycle V), resulting in total sugar of 41.3–42.6 $g{\cdot}L^{-1}$ for each cycle. RS hydrolysates (Cycle I and V) was utilized in butanol fermentation by *Clostridium saccharobutylicum* DSM 13864, achieving butanol titer and yield of 9.5 $g{\cdot}L^{-1}$ and 0.25 $g{\cdot}g^{-1}_{total\ sugar}$, similar to those of glucose medium. This study demonstrated the feasibility of this newly developed biomass pretreatment by dihydrogen bonding DES featuring cellulase recycling and high butanol yield.

1. Introduction

The demand for non-renewable energy sources such as oil, gas and coal is ever increasing in modern society. Our known fossil fuel deposits will be depleted in 40–50 years at current consumption rate. Butanol, is one of the most promising biofuels, possessing superiorities such as higher hydrophobicity and energy density, lower corrosivity, and easier miscibility with gasoline in comparison to ethanol [1]. Besides that,

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butanol is an important organic solvent and chemical reagent with widespread applications in paint, plastics and organic synthesis [2]. Biobutanol, produced from lignocellulosic biomass by solventogenic clostridia or *Escherichia coli* and *Saccharomyces cerevisiae* engineered with heterogeneously solventogenic pathways, is regarded as a promising alternative to chemical synthesis of butanol, concerning the exhausted fossil fuels and environmental pollution [3–5].

Rice straw (RS) is the most abundant lignocellulosic biomass in China with an annual production ranging between 180 and 270 million tons [6], which could be potentially transformed into biofuels. Traditionally, RS is burnt in the field to enhance soil fertility, whereas has caused serious air pollution. The main components of RS are cellulose, hemicellulose and lignin. Among them, cellulose and hemicellulose are main sources of fermentable sugars, consisting 66–75% dry weight of lignocellulosic raw materials [7], while the lignin content is lower than other feedstocks such as wheat straw [8]. Therefore, conversion of cheap RS into biofuels is a promising strategy to alleviate current energy crisis and environmental pollution. Cellulose is a chain-like polymer in which glucosides are linked by β-1,4-glycosidic linkages. A plurality of cellulose molecules are arranged in parallel to form small fibers, which constitute plant cellulose in the end. Lignin is composed of phenylpropane units connected with carbon-carbon and carbon-oxygen-carbon bonds, forming a network structure. Lignin, as the supporting skeleton, is crossly linked with hemicellulose between the fine fibers of the cell wall, which could reinforce the cell wall of the woody tissue and enhance the adhesion between cells. However, the presence of lignin and hemicellulose reduces the accessibility of cellulase to cellulose, resulting in lower conversion efficiency of rice straw [9]. Therefore, the tight structure of lignocellulose must be broken to fluff out the RS, increase the degree of crystallinity of cellulose, and further improve the efficiency of enzymatic hydrolysis.

Different pretreatment methods, such as physical (mechanical crushing, high temperature and pressure) [10], chemical (strong acids or bases) [11] and biological (microbial/enzymatic decomposition of lignin and lignocellulose) [12], have been developed and applied in recent years [13]. The applications of physical pretreatment methods are limited due to its high energy demand and equipment cost. Chemical methods are usually effective in the pretreatment, however, are highly corrosive and environmentally unfriendly. Compared with chemical and physical pretreatments, biological methods has a number of advantages such as mild reaction, less energy consumption, simple equipment and minimal environmental pollution. Whereas, biological methods often require longer treatment period [14]. Ionic liquids (ILs) has emerged as a promising “green solvents” to improve the biomass utilization [15]. In addition, they are also referred to as “designer solvents” because the anion and cations of ILs can be selected and combined as desired [16–18]. They usually possess high thermal and chemical stability, non-flammability, design ability, non-volatility, recyclability and excellent solvent properties to dissolve solutes of varying polarity [19]. ILs have been widely used in electrochemistry, chemical conversion, material separation, biocatalysis and transformation. Moreover, ILs exhibit good dissolving property towards lignin [20,21]. In recent years, efforts are devoted to exploring the application value of ionic liquids in biomass dissolving and pretreatment. It is widely acknowledged that deep eutectic solvents (DESs) are a new class of ILs based on eutectic mixtures of halide salt or another hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD), which share the characteristics of low melting point, easy biodegradability and high preparation yield, in addition to all the common properties of ILs [22]. Therefore, DES has been introduced in pretreatment of lignocellulosic biomass in recent years. Choline acetate ([Ch][OAc]) was found to be a biocompatible alternative to [Emim][OAc] for lignocellulosic material pretreatment. The cellulose saccharification ratio of [Ch][OAc] pretreatment at 110 °C for 16 h was 100.6%, which was almost comparable with that of [Emim][OAc] [23]. Corncob residues were pretreated with three different DES systems: choline chloride and glycerol, choline chloride and imidazole, choline chloride and urea. A total of 41 g fermentable sugars (27 g glucose and 14 g xylose) could be recovered from 100 g corncob, representing 76% (86% and 63%) of the initially available carbohydrates [24].

In our previous report, a DES named as ChCl:formic acid (forline) had been synthesized and applied in pretreatment of corn straw. *Clostridium saccharobutylicum* could efficiently utilize lignocellulosic hydrolysates, especially xylose and arabinose, for butanol fermentation. Utilizing the corn straw hydrolysate, the butanol titer, yield and productivity of 5.63 $g{\cdot}L^{-1}$, 0.17 $g{\cdot}g^{-1}_{\text{total sugar}}$ and 0.12 $g{\cdot}L^{-1}{\cdot}h^{-1}$ were obtained with *C. saccharobutylicum* DSM 13864 [25]. In this study, several dihydrogen-bonding DESs were synthesized using formic acid, acetic acid, glycerol and urea as HBD and choline chloride as HBA. These dihydrogen-bonding DESs were supposed to have superiority in pretreatment of lignocellulosic biomass than the monohydrogen-bonding DESs. The application potential of dihydrogen-bonding DESs in the pretreatment of RS was systematically evaluated. At the same time, to reduce cellulase cost, a cellulase reutilization process was developed in the hydrolysis of pretreated RS and the corresponding hydrolysates were subjected to biobutanol fermentation.

2. Material and methods

2.1. Strains, feedstock, and chemicals

C. saccharobutylicum DSM 13864 was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures). In order to induce sporulation, it was cultivated in Reinforced Clostridia Medium (RCM) at 37 °C for 7 days and stored at room temperature. Spore suspension (10%, *v/v*) was inoculated in 12 mL sterile RCM and then placed in a desiccator evacuated to a vacuum level of 0.065 MPa for anaerobic condition. Afterwards, the culture was cultivated at 37 °C for 12–18 h and regarded as the seed medium [26].

Rice straw (RS) was obtained from a farm in Suining, Sichuan Province, China. RS was initially dried at 60 °C in oven for one day, and milled by grinder. Then, the milled RS was passed through a 380 μm sieve, and dried at 60 °C for 24 h before use. The particle size distribution of the RS 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.

Choline chloride (ChCl, 98% purity) was obtained from Shanghai Titan Chem Co., Ltd. Cellulase was a generous gift from Vland Biotech Co Ltd. (Qingdao, China). All other chemicals were of reagent or analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd.

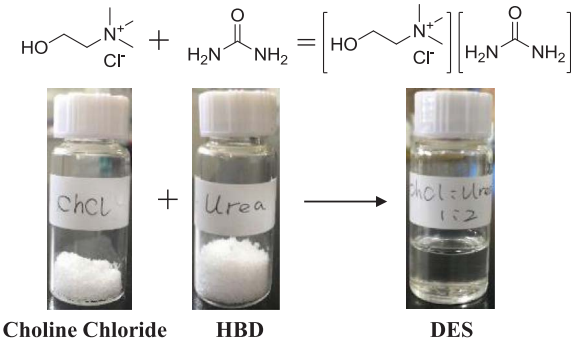
2.2. Synthesis of DES with single or double hydrogen bond donors

Chloline chloride as HBA was mixed with formic acid, acetic acid, glycerol and urea as HBDs to form forline, aceline, reline, glyceline, foraceline, forglyceline, forreline, aceglyceline and acereline (Table 1). Then, the mixtures were heated up to 30 °C or 60 °C and stirred at 150 rpm in a conical flask with a plug to reduce evaporation until formation of homogenous transparent liquid as previously reported [27]. Afterwards, the synthesized DESs were heated up to around 80 °C for 12 h to remove the unreacted free acids. Then, they were stored in a vacuum desiccator for further use.

2.3. Comparison on pretreatment of RS with DESs

Pretreatment of RS with DESs was performed with 10 g RS in 200 g nine DESs at 130 °C in oil bath for 2 h. Afterwards, appropriate volume of hot deionized water (85 °C) was added for regeneration of cellulose. Then, the resultant pretreated RS was subjected to washing, filtration, drying to constant weight, and sieving through a 380 μm griddle. The pretreated RS was stored at 4 °C for enzymatic hydrolysis. The resultant RS pretreated by foraceline was designated as $RS_{\text{foraceline}}$, and the others

Table 1
Synthesis of choline chloride based DESs with different hydrogen bond donors.



DES	HBD[a]	ChCl:HBD [molar ratio]	Temp. [°C]	Time [h]
Reline	Urea	1:2	60	2
Glyceline	Glycerol	1:2	60	2
Forline	Formic acid	1:2	30	2
Aceline	Acetic acid	1:2	30	2
Foraceline	Formic acid, Acetic acid	1:1:1	30	2
Forglyceline	Formic acid, Glycerol	1:1:1	30	2
Forreline	Formic acid, Urea	1:1:1	30	2
Aceglyceline	Acetic acid, Glycerol	1:1:1	30	2
Acereline	Acetic acid, Urea	1:1:1	30	2

[a] HBD: hydrogen bond donor.

were in the same abbreviation unless otherwise stated.

Enzymatic hydrolysis mixture consisted of 1 g RS pretreated by DESs dispersing in 10 mL citrate buffer (50 mM, pH 4.8), 100 μL ampicillin (1 g·L^{-1}) and 50 FPU cellulase (142 ± 1.1 FPU/g) in a 50-mL flask. The enzymatic hydrolysis mixtures were incubated in a water bath at 50 °C and 150 rpm for 48 h. Samples (400 μL) were withdrawn at 6, 12, 24 and 48 h, and then 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 using DNS method as previously described [28]. All the enzymatic hydrolysis reactions were separately performed in triplicate.

2.4. Optimization of two-stage pretreatment

2.4.1. Effects of operation modes of foraceline and Na_2CO_3 on the RS pretreatment

Two-stage pretreatment of RS by foraceline and Na_2CO_3 was optimized in four different operation modes ($Comb_{AD1}$, $Comb_{DA1}$, $Comb_{AD2}$, and $Comb_{DA2}$). Specifically, $Comb_{AD1}$: RS was soaked in 1% (w/v) Na_2CO_3 for 24 h at 1:10 ratio under room temperature, then pretreated by foraceline at 140 °C for 2 h; $Comb_{DA1}$: same foraceline and Na_2CO_3 pretreatments were performed in reverse order of $Comb_{AD1}$; $Comb_{AD2}$: RS was pretreated by 1% (w/v) Na_2CO_3 for 1 h at 1:10 ratio and 140 °C, then pretreated by foraceline at 140 °C for 2 h; $Comb_{DA2}$: same foraceline and Na_2CO_3 pretreatments were performed in reverse order of $Comb_{AD2}$. The resultant RSs were designated as $RS_{CombAD1}$, $RS_{CombAD2}$, $RS_{CombDA1}$ and $RS_{CombDA2}$. Untreated RS (RS_U) or RS treated with single reagent (foraceline for $RS_{foraceline}$ or Na_2CO_3 for RS_{Na2CO3}) was used as controls.

2.4.2. Effects of conditions on RS pretreatment

Effects of solid to liquid ratio, temperature and pretreatment time on the pretreatment of RS with foraceline were performed as above mentioned, except for solid ratios of 1:7, 1:8, 1:10 and 1:12, temperatures of 110 °C, 120 °C, 130 °C and 140 °C, pretreatment time of 0.5 h, 1.0 h, 2.0 h and 3.0 h. The $RS_{foraceline}$ was obtained by washing, filtration, drying to constant weight, sieving through a 380 μm griddle, and stored at 4 °C for enzymatic hydrolysis.

Effect of concentration of Na_2CO_3 solution, temperature and pretreatment time on the pretreatment of $RS_{foraceline}$ with Na_2CO_3 were performed as above mentioned, except for concentrations of Na_2CO_3 solution of 0.0%, 0.1%, 0.3%, 0.5%, 1.0%, 2.0%, 5.0%, 8.0% and 10.0%, temperatures of 60 °C, 80 °C, 100 °C, 120 °C and 140 °C, pretreatment time of 0.5 h, 1.0 h, 1.5 h, 2 h and 3 h. The $RS_{CombDA2}$ was obtained by washing, filtration, drying to constant weight, sieving through a 380 μm griddle, and stored at 4 °C for enzymatic hydrolysis.

All the experiments were carried out in triplicate.

2.5. Optimization on the enzymatic hydrolysis of pretreated RS

Effects of cellulose dosage and substrate loading on the enzymatic hydrolysis of $RS_{CombDA2}$ were implemented as above described for 72 h, except for cellulase dosages of 10 FPU·$g^{-1}_{total\ solid}$, 30 FPU·$g^{-1}_{total\ solid}$, 50 FPU·$g^{-1}_{total\ solid}$ and 70 FPU·$g^{-1}_{total\ solid}$, substrate loadings of 1:7, 1:8, 1:10 and 1:12. Samples (300 μL) were collected at 6, 12, 24, 36, 48, 60 and 72 h, and centrifuged at 12,000 × *g* for 10 min. Supernatants (100 μL) were added with 0.4% H_2SO_4 (450 μL, w/w) to stop the hydrolysis reactions. The concentration of reducing sugars was determined using HPLC analysis as previously reported [28]. All the enzymatic hydrolysis reactions were separately performed in triplicate.

2.6. Recovery of solid-bound cellulases

Cellulases adsorbed to the $RS_{CombDA2}$ were reutilized. Effect of cellulase dosage on the hydrolysis of $RS_{CombDA2}$ in the Cycle II was investigated. Enzyme hydrolysis of Cycle II was performed at 1:10 substrate loading and 50 °C, enzyme dosages were 0%, 30%, 60%, 90% and 100% of the optimized enzyme dosage (100% = 50 FPU·$g^{-1}_{total\ solid}$). After hydrolysis for 24 h, the hydrolysis mixtures were terminated by addition of 0.4% H_2SO_4 and subjected to reducing sugars analysis.

For cellulase reutilization, every cycle of enzymatic hydrolysis was conducted in a 50 mL conical flask with stopper, consisted of 1 g $RS_{CombDA2}$ dispersing in 10 mL citrate buffer (50 mM, pH 4.8), 100 μL ampicillin (1 g·L^{-1}) and cellulase (for Cycle I: 50 FPU·$g^{-1}_{total\ solid}$, and at descending of 10% for each cycle). The hydrolysis mixtures were incubated at 50 °C and shaken at 150 rpm for 24 h. Samples were withdrawn from the mixture at 6 h, 12 h and 24 h and subjected for the determination of total sugar concentrations. At the end of each cycle, the enzymatic hydrolysis mixture was centrifuged at 12,000 × *g* for 10 min. The liquid fraction was collected in 100 mL anaerobic bottle and stored at 4 °C for fermentation. The cellulase absorbed to the residual $RS_{CombDA2}$ in the precipitant were recycled to the next enzymatic hydrolysis cycle, and the fresh $RS_{CombDA2}$ and cellulase at 10% descending of optimized enzyme dosage were supplemented and carried out as above mentioned. The reutilization of cellulase was continuously operated for five cycles.

2.7. Methods for characterization of rice straw

2.7.1. Component analysis

Analysis of cellulose, hemicellulose, lignin and ash in RS_U, $RS_{foraceline}$ and $RS_{CombDA2}$ were carried out as previously reported [28]. Removal rate of hemicellulose and lignin was calculated according to the formulas as following:

$$\text{Hemicellulose removal (\%)} = \left(1 - \frac{\text{Hemicellulose in pretreated RS}}{\text{Hemicellulose in untreated RS}} \times \text{solid yield}\right) \times 100\%$$

$$\text{Delignification (\%)} = \left(1 - \frac{\text{Lignin in pretreated RS}}{\text{Lignin in untreated RS}} \times \text{solid yield}\right) \times 100\%$$

2.7.2. SEM analysis

Scanning electron microscopy (5.0 kV, ×1200 Hitachi S-4800, Japan) analysis was operated to observe the surface morphological features of RS before and after pretreatment.

2.7.3. FTIR analysis

Chemical structure of the pretreated RS was detected with Fourier Transform Infrared Spectroscopy (FTIR, Nicolet IS50). FTIR spectra was obtained at spectral range of 600–1800 cm^{-1}.

2.7.4. XRD analysis

X-ray diffractometer (XRD) was used to measure the crystallinity of the pretreated and unpretreated RS, using a D/max 2500 PC diffractometer with Cu/Ka radiation source (Rigaku Corporation, Tokyo, Japan). It was operated at a voltage of 60 kV and a current of 300 Ma with a scanning speed of 0.02°/min and the 2θ range from 5° to 40°. Crystallinity index (CrI) was calculated as following [29]:

$$\text{CrI}\ (\%) = \frac{I_{002} - I_{am}}{I_{002}} \times 100\%$$

I_{002} and I_{am} imply the intensities of the peak at near 21.4°and 16.0° respectively.

2.8. Biobutanol fermentation by C. saccharobutylicum DSM 13864

The RS hydrolysates Cycle I and V were collected and concentrated to total sugar of around 50 $g{\cdot}L^{-1}$. Then, other components of the fermentation medium were added, including corn steep liquor (CSL), 10 $g{\cdot}L^{-1}$; $CaCO_3$, 4 $g{\cdot}L^{-1}$; $(NH_4)_2SO_4$, 2 $g{\cdot}L^{-1}$; K_2HPO_4, 0.5 $g{\cdot}L^{-1}$; $MnSO_4$-H_2O, 0.01 $g{\cdot}L^{-1}$. The fermentation medium containing 50 $g{\cdot}L^{-1}$ of glucose instead of hydrolysates was used as control. Afterwards, the pH of the medium was adjusted to 6.5 with 4 M NaOH and autoclaved at 115 °C for 20 min. The fermentation medium in a 100-mL anaerobic bottle was inoculated with 10% of actively growing strain, and anaerobically incubated at 37 °C in a desiccator (0.065 MPa) [28]. Samples were regularly withdrawn and the ABE contents were analyzed by gas chromatography (6890N; Agilent Technologies, Wilmington, DE, USA) according to previously described method [26]. Butanol fermentation with hydrolysates medium and glucose medium were all carried out in triplicate.

3. Results and discussion

3.1. Comparison of deep eutectic solvents for RS pretreatment

The eutectic solvent of choline chloride and formic acid (ChCl:formic acid, 1:2, forline) played an important role in the pretreatment of corn straw [25]. Here, several deep eutectic solvents were synthesized using single hydrogen bond donor or double hydrogen bond donors and choline chloride as the hydrogen bond acceptor, including forline (ChCl:formic acid, mole ratio of 1:2), aceline (ChCl:acetic acid, 1:2), glyceline (ChCl:glycerol, 1:2), reline (ChCl:urea, 1:2), foraceline (ChCl:formic acid:acetic acid, 1:1:1), forglyceline (ChCl:formic acid:glycerol, 1:1:1), forreline (ChCl:formic acid:urea, 1:1:1), aceglyceline (ChCl:acetic acid:glycerol, 1:1:1) and acereline (ChCl:acetic acid:urea, 1:1:1) (Table 1). All the DESs were synthesized by gently mixing the choline chloride and corresponding HBDs at 30 °C or 60 °C until formation of transparent and homogenous liquid, generally for 2 h, according to previously reported protocol [27]. All DESs were tested in pretreatment of rice straw at 130 °C for 2 h, and solid-liquid ratio of 1:20.

The concentrations of total sugar after enzymatic hydrolysis of RS pretreated by four monohydrogen-bonding and five dihydrogen-bonding DESs were determined using the DNS method and illustrated in Fig. 1A and Fig. 1B respectively. It can be seen from Fig. 1A that the highest total sugar concentration of 17.7 $g{\cdot}L^{-1}$ was obtained using forline with initial hydrolysis rate of 1.33 $g{\cdot}L^{-1}{\cdot}h^{-1}$. In spite of the highest hydrolysis rate was 1.70 $g{\cdot}L^{-1}{\cdot}h^{-1}$ by pretreatment with aceline, the final total sugar was only 12.8 $g{\cdot}L^{-1}$ total sugar, 27.7% lower than that of forline. The final sugar concentrations of formic acid aqueous solution (39.7% in water) and acetic acid aqueous solution (46.2% in water) as controls were 13.8 $g{\cdot}L^{-1}$ and 6.86 $g{\cdot}L^{-1}$, 21.8% and 43.8% lower than that of forline and aceline, indicating the effectiveness of forline and aceline in the pretreatement of RS. The total sugar concentrations released from $RS_{glyceline}$ and RS_{reline} were 5.93 $g{\cdot}L^{-1}$ and 6.07 $g{\cdot}L^{-1}$, similar to 4.18 $g{\cdot}L^{-1}$ of RS_U.

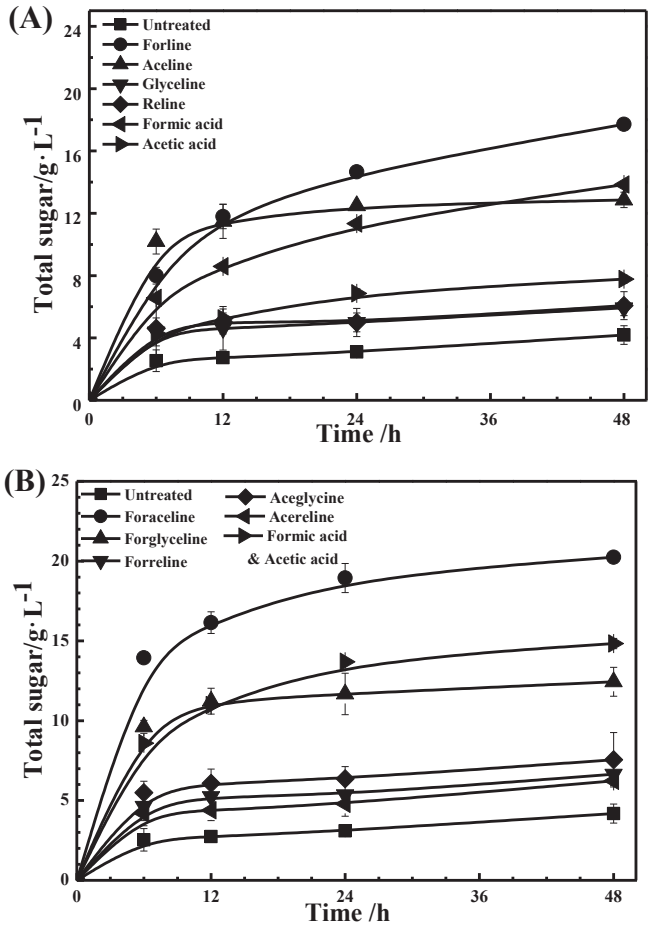


Fig. 1. Comparison of various deep eutectic solvents with choline chloride as the hydrogen bond receptor and different hydrogen bond donors in the pretreatment of rice straw. (A) Single hydrogen bond donor. Formic acid aqueous solution and acetic acid aqueous solution were regarded as controls. (B) Double hydrogen bond donors. Formic acid and acetic acid aqueous solution was regarded as control.

Five DESs with double hydrogen bond donors were synthesized and applied in RS pretreatment (Fig. 1B). Remarkable effect was found in pretreatment with foraceline. The total sugar concentration after hydrolysis of 48 h and hydrolysis rate of initial 6 h were 21.5 $g{\cdot}L^{-1}$ and 2.32 $g{\cdot}L^{-1}{\cdot}h^{-1}$, which were 1.14- and 1.74-fold of forline pretreatment, 1.37- and 1.57-fold of aceline pretreatment, 1.36- and 1.62-fold of the pretreatment with formic acid and acetic acid aqueous solution (43.2% in water). All the results proved the positive effect of dihydrogen-bonding foraceline in comparison with the monohydrogen-bonding forline, aceline and the corresponding controls. In order to get insight into the role of foraceline in RS pretreatment, component analysis of $RS_{foraceline}$ was performed and shown in Table 2. The cellulose content was 50.8%, 71.0% higher than in RS_U. The hemicellulose and total lignin decreased from 4.9% to 3.1%, 30.4% to 27.2%, with the hemicellulose removal rate and delignification rate of 60.1% and 43.6%.

Table 2
Compositions of untreated and pretreated rice straw.

Component [%]	Rice straw pretreated by different methods						
	Untreated	Foraceline	Na_2CO_3	$Comb_{AD1}$	$Comb_{DA1}$	$Comb_{AD2}$	$Comb_{DA2}$
Cellulose	29.7 ± 0.2	50.8 ± 1.5	37.4 ± 0.2	55.9 ± 0.3	57.2 ± 1.6	49.6 ± 0.9	58.6 ± 0.8
Hemicellulose	4.9 ± 0.9	3.1 ± 0.1	6.3 ± 0.0	2.2 ± 0.1	3.1 ± 0.0	3.2 ± 0.0	2.9 ± 0.3
Hemicellulose removal	–	60.1	17.2	76.1	75.2	79.7	83.1
Acid soluble lignin	1.7 ± 0.3	0.9 ± 0.0	1.1 ± 0.1	0.6 ± 0.0	0.7 ± 0.5	0.6 ± 0.0	0.5 ± 0.0
Acid insoluble lignin	28.7 ± 0.3	26.3 ± 0.15	22.1 ± 0.3	22.1 ± 0.3	25.6 ± 0.2	22.0 ± 1.1	21.0 ± 0.2
Delignification	–	43.6	50.8	65.8	67.4	76.8	79.8
Ash	9.4 ± 1.2	11.6 ± 1.0	6.4 ± 0.1	11.9 ± 0.9	9.7 ± 0.6	9.1 ± 0.5	11.9 ± 0.1
Others	25.4 ± 0.1	7.3 ± 0.7	26.7 ± 0.7	7.3 ± 1.2	3.7 ± 0.1	15.5 ± 0.5	5.1 ± 0.2

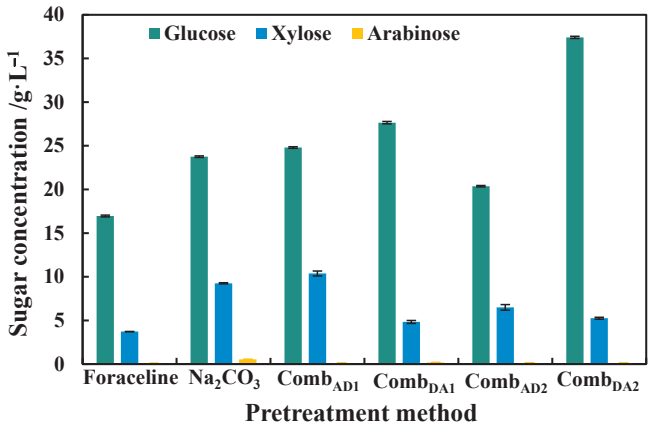


Fig. 2. Effect of combinatorial pretreatment employing foraceline and Na_2CO_3.$Comb_{AD1}$: RS was soaked in 1% (w/v) Na_2CO_3 for 24 h at 1:10 ratio under room temperature, then pretreated by foraceline at 140 °C for 2 h; $Comb_{DA1}$: same foraceline and Na_2CO_3 pretreatments were performed in reverse order of $Comb_{AD1}$; $Comb_{AD2}$: RS was pretreated by 1% (w/v) Na_2CO_3 for 1 h at 1:10 ratio and 140 °C, then pretreated by foraceline at 140 °C for 2 h; $Comb_{DA2}$: same foraceline and Na_2CO_3 pretreatments were performed in reverse order of $Comb_{AD2}$.

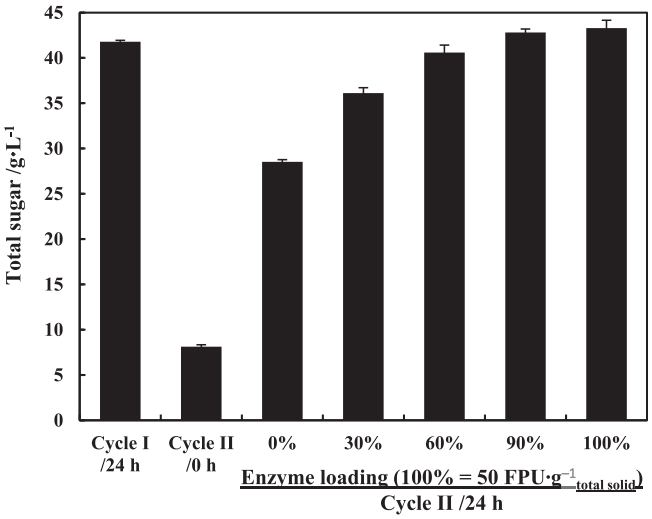


Fig. 3. Optimization of enzyme dosages for cellulase recycle. The enzyme loading for Cycle I was 50 FPU·$g^{-1}_{total\ solid}$ and regarded as 100%.

Component analysis of $RS_{forline}$ displayed increased cellulose of 54.5%, hemicellulose removal of 66.2% and delignification rate of 23.8% [25]. Pretreatment with foraceline showed higher advantages in the delignification rate than with forline. Considering the notable effect in improving the cellulase accessibility of RS, the application potential of foraceline in pretreatment of RS and biobutanol fermentation was investigated.

3.2. Establishment of two-stage pretreatment using foraceline and sodium carbonate

It is widely acknowledged that the mechanisms of alkaline in pretreatment of lignocellulosic biomass are removing lignin and loosening lignin structures, thereby reducing biomass recalcitrance and enhancing the enzymatic hydrolysis efficiency [30]. To further improve the pretreatment effect in enhancing the cellulase accessibility and delignification rate, a two-stage pretreatment using foraceline and alkaline reagents was proposed. Several alkali salts such as sodium carbonate (Na_2CO_3), sodium sulfide (Na_2S), sodium phosphate (Na_3PO_4), which were weak alkaline and reported as feasible regents for biomass pretreatment because of their recuperability and less corrosivity [29], were evaluated in the pretreatment of RS. Enzymatic hydrolysis experiments revealed that Na_2CO_3 was the most effective pretreatment reagent (data not shown). Consequently, a two-stage pretreatment in combination of foraceline and Na_2CO_3 was developed in the pretreatment of RS.

The foraceline-Na_2CO_3 ($Comb_{DA1}$ and $Comb_{DA2}$) and Na_2CO_3-foraceline ($Comb_{AD1}$ and $Comb_{AD2}$) pretreatments in reverse sequences were compared (Fig. 2). Comparison between $Comb_{AD1}$ and $Comb_{DA1}$ or $Comb_{AD2}$ and $Comb_{DA2}$, Na_2CO_3 pretreatment ahead of foraceline resulted in higher xylose while foraceline ahead of Na_2CO_3 produced higher glucose, which was in consistence with the single foraceline and Na_2CO_3 pretreatment, indicating a synergistic effect of the two pretreatment methods. Among these combinational pretreatments, $Comb_{DA2}$ showed distinct advantages in RS pretreatment, by which the highest glucose and total sugar of 37.3 g·L^{-1} and 42.7 g·L^{-1} were attained. It indicates that foraceline pretreatment followed by Na_2CO_3 (1%) pretreatment is preferable for further enzymatic hydrolysis of RS. Moreover, the impacts of pretreatment on compositions of RS were summarized in Table 2. Untreated rice straw consisted of 29.7% (w/w) cellulose, 4.9% hemicellulose, 28.7% acid-insoluble lignin, 1.7% acid-soluble lignin, and 9.4% ash on a dry weight basis. Alkaline pretreatment can cause degradation, reallocation and condensation of lignin and changes in the crystalline state of cellulose. Among them, RS pretreated by $Comb_{DA2}$ showed the highest lignin and hemicellulose removal of 79.8% and 83.1%, respectively, which was in accordance with the results of enzymatic hydrolysis (Fig. 2). It is speculated that Cl^- anion in foraceline can effectively break the comprehensive hydrogen-bond networks existing in lignocellulose and remove a large proportion of hemicellulose [31]. Then the surface of RS pretreated by Na_2CO_3 became more bouffant and polyporous, which made it more accessible to cellulase and thereby enhanced the sugar yield.

3.3. Optimization of $RS_{CombDA2}$ pretreatment conditions

Considering the notable effect of this two-stage pretreatment, some operational conditions including chemical dosage, temperature, time

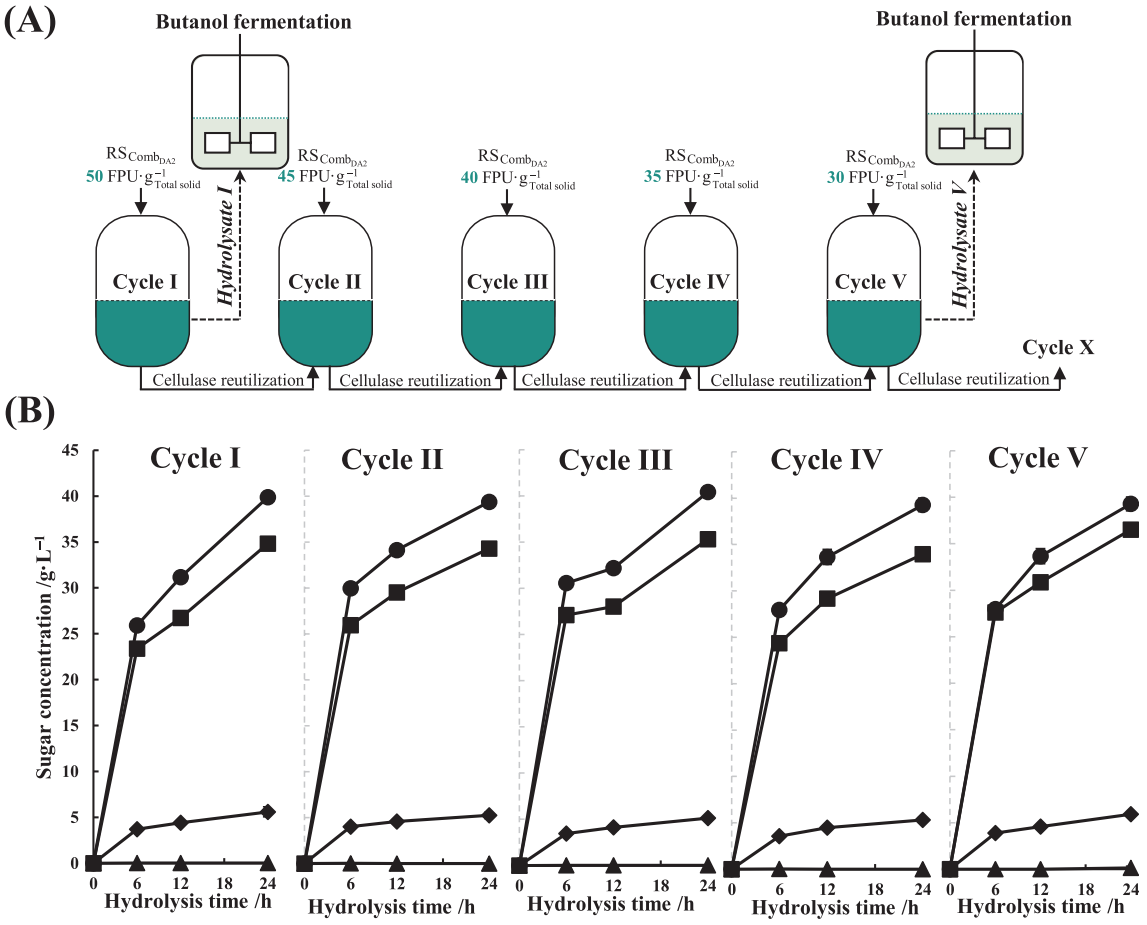


Fig. 4. Reutilization of cellulase and hydrolysis of rice straw pretreated by foraceline and Na_2CO_3. (A) scheme of the reutilization of cellulase; (B) enzymatic hydrolysis result of $RS_{CombDA2}$. Cycle (●): total sugar; square (■): glucose; diamond (♦): xylose; triangle (▲): arabinose. Enzymatic hydrolysis was performed at solid-liquid ratio of 1:10 and 50 °C for 24 h, and with different enzyme loading, for Cycle I: 50 FPU·$g^{-1}_{total solid}$, Cycle II: 45 FPU·$g^{-1}_{total solid}$, Cycle III: 40 FPU·$g^{-1}_{total solid}$, Cycle IV: 35 FPU·$g^{-1}_{total solid}$, Cycle V: 30 FPU·$g^{-1}_{total solid}$.

and solid-liquid ratio in the foraceline and Na_2CO_3 pretreatments were optimized to achieve higher total sugar concentration and environmental benignity (Figs. S1 & S2). For foraceline, total sugar contents were increased along with the increasing of RS to foraceline ratios. In view of the mass transfer efficiency, RS to foraceline ratio of 1:10 was selected. Higher temperature was beneficial to the foraceline pretreatment, in which the highest sugar content was obtained at 140 °C. Two hours pretreatment with foraceline was suitable, while further elongation time to 3 h resulted in loss of total sugar. With regard to Na_2CO_3, obviously, with the increasing of Na_2CO_3 dosage, the total sugar increased steadily from 27.3 g/L to 49.2 g/L, however higher addition of Na_2CO_3 caused severe corrosion. It can be seen that 0.5% Na_2CO_3 was adequate for RS pretreatment. Effects of temperature and incubation time on the Na_2CO_3 pretreatment were investigated, and 80 °C and 1.5 h incubation were returned to be the optimum conditions. In summary, RS to foraceline ratio of 1:10 and temperature of 140 °C for 2 h were determined to be the optimum conditions for foraceline pretreatment, and 0.5% (w/v) Na_2CO_3 at 80 °C for 1.5 h were the optimum conditions for Na_2CO_3 pretreatment. Under the optimized conditions, the highest total sugar and glucose concentrations of 41.9 g·L^{-1} and 38.8 g·L^{-1} were obtained.

3.4. Physical characterization of pretreated RS

Physical characterization of RS_U, $RS_{foraceline}$ and $RS_{CombDA2}$ was investigated to get insight into the changes of RS and mechanisms of pretreatment. SEM images of RS_U, $RS_{foraceline}$ and $RS_{CombDA2}$ and RS residues after enzyme hydrolysis were shown in Fig. S4. For RS_U, the rigid, uneven and compact structure was clearly visible (Fig. S4A). The outer layer of the RS is mostly composed of lignin, ash, and hemicellulose, which enclose the interior cellulose fibers and hinder the enzymatic access to cellulose during saccharification [32]. Compared with raw RS, some evident changes were observed in the outmost surface of $RS_{foraceline}$, which showed apparent abrasion and splitting of fibers, as well as some delamination and scaling due to the decomposition of partial hemicellulose (Fig. S4B). For $RS_{CombDA2}$, the surface became looser and had some sunk areas, as well as more layers and shrinkage, representing more serious damage of RS surface (Fig. S4C). This observation is in accordance with the high delignification and hemicellulose removal in $RS_{CombDA2}$ (Table 2), which is advantageous for enzymatic hydrolysis. The SEM image of enzyme hydrolyzed RS showed that the original compact structures have transformed into a number of long strips, indicating that the structure of cellulose was severely destroyed by cellulase (Fig. S4D). The DES and Na_2CO_3 two-stage pretreatment was highly conducive to the cellulase hydrolysis of RS.

Moreover, the FTIR spectra of the untreated and pretreated RS were also compared (Fig. S5). The positions of absorption peaks were assigned to chemical components according to related data [33,34]. The characteristic absorption peaks of cellulose were at 895–900 cm^{-1} associated with the β-glycosidic bond, connecting with the removal of the relevant amorphous cellulose, especially in hemicellulose. The bond at around 1037 cm^{-1} was attributed to C—O stretching from guaiacyl-type lignin, hemicellulose, or cellulose. In comparison with untreated RS, the absorptions of corresponding bands (1140 and 1087 cm^{-1}) in these regions were reduced in the pretreated substrate and $RS_{CombDA2}$ was

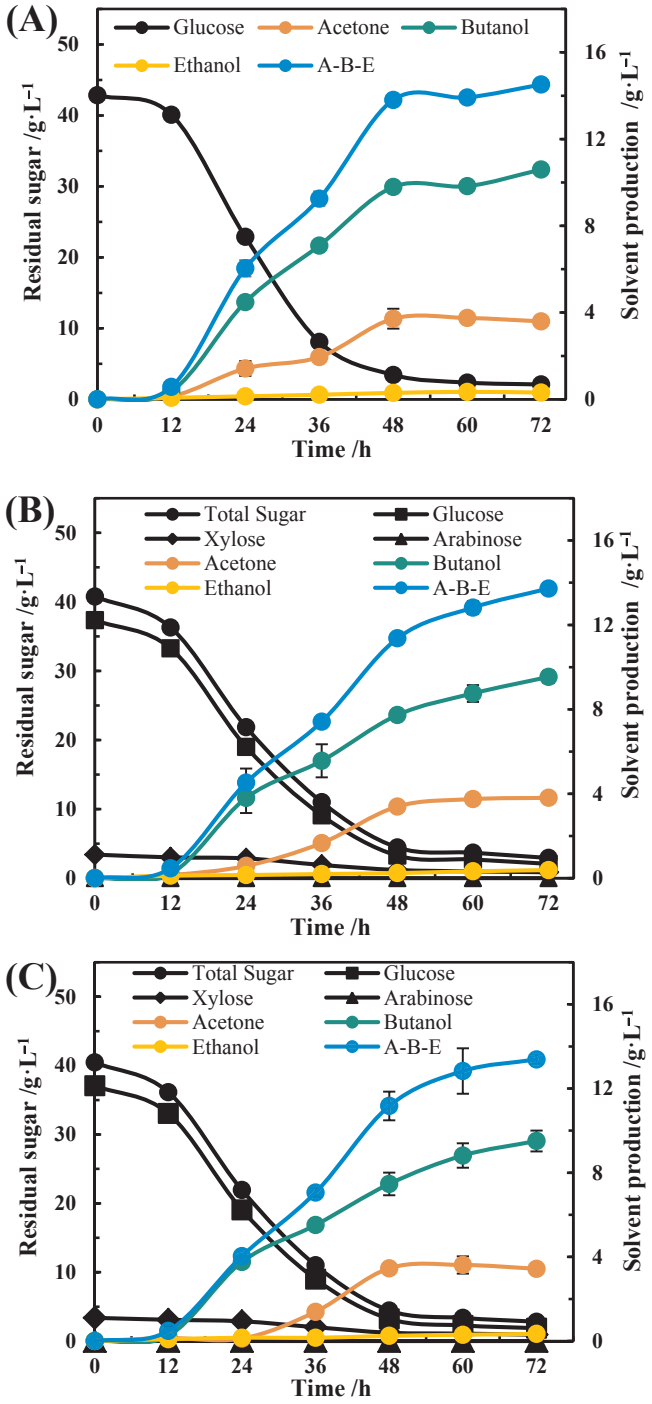


Fig. 5. Butanol fermentation by *C. saccharobutylicum* DSM 13864 using glucose and RS hydrolysates medium. (A) Glucose; (B) Hydrolysate I (Cycle I); (C) Hydrolysate V (Cycle V). The fermentation medium and conditions were as follows: total sugar 50 g·L^{-1}; 10 g·L^{-1} CSL; 4 g·L^{-1} $CaCO_3$; 2 g·L^{-1} $(NH_4)_2SO_4$; 0.5 g·L^{-1} K_2HPO_4; 0.01 g·L^{-1} $MnSO_4·H_2O$, 50 mL, pH 6.5, 37 °C, inoculum size of 10% (v/v).

more, indicating the removal of lignin. The band about 1315 cm^{-1} could be caused by the C—H bending vibrations in cellulose and hemicellulose, suggesting the removal of a large amount of amorphous cellulose. The decrease or even disappearance of the peak at 1730 cm^{-1} is due to the change of carboxyl groups in hemicellulose, indicating dislodgement of partial hemicellulose. As shown in Table 2, for $RS_{foraceline}$ and $RS_{CombDA2}$, the hemicellulose removals were 60.1% and 83.1%, respectively. These results substantiate the components wrapped around the cellulose were effectively removed, which improved the efficiency of cellulase hydrolysis.

To further evaluate the crystallinity of the pretreated RS, XRD profiles of RS_U, $RS_{foraceline}$ and $RS_{CombDA2}$ were recorded (Fig. S6). Compared with RS_U, no new peaks appeared in $RS_{foraceline}$ and $RS_{CombDA2}$, indicating that pretreatment did not destroy the crystal structure of cellulose. As depicted in Fig. S6, native biomass exhibited diffraction peaks near 15–16°, 22.5°, and 35° (2θ). These peaks remained in their original position, but the peak shape became sharper for pretreated RS, especially for $RS_{CombDA2}$. The crystallinity of RS_U, $RS_{foraceline}$ and $RS_{CombDA2}$ were 47.9%, 55.8% and 57.5%, respectively. The increased crystallinity in pretreated biomass implied the removal of amorphous, noncellulose components, such as lignin and hemicellulose, which was also in agreement with the high glucose concentration as illustrated in Table 2.

3.5. Reutilization of solid-bound cellulase

Cellulase accounts for a large portion of cost in the saccharification and application of lignocellulosic biomass in biofuels. Cellulase absorb to the biomass and hydrolyze the cellulose. Generally at the end of hydrolysis procedure, the cellulase absorbed to the biomass would be discarded with biomass residues. To reduce the cellulase dosage and save the cellulase cost, a reutilization of absorbed cellulase process was developed. First of all, effects of cellulase dosage and substrate loading on the saccharificaiton of $RS_{foraceline}$ were investigated (Fig. S6). 50 FPU·$g^{-1}_{total\ solid}$ of cellulase dosage and 10% of substrate loading for 24 h of hydrolysis time were returned as the optimal saccharification conditions.

Experiments were carried out to optimize the enzyme loading for subsequent cycle II to obtain similar sugar concentration as Cycle I (Fig. 3). At the starting of cycle II, there was 8.12 g·L^{-1} total sugars attached to the un-hydrolyzed residues. Then, different dosages of cellulase in Cycle II from 0%, 30%, 60% 90% and 100% of the optimized cellulase dosage (50 FPU·$g^{-1}_{total\ solid}$) were added in. As illustrated Fig. 3, 28.5 g·L^{-1} total sugar was obtained without additional enzymes after 24 h of hydrolysis, suggesting residual cellulase from Cycle I functioned well in Cycle II. Addition of 90% cellulase (45 FPU·$g^{-1}_{total\ solid}$) of the optimized cellulase dosage, the total sugar could reach 42.8 g·L^{-1}, a littler higher than that of Cycle I. It was confirmed that the amount of cellulase supplemented per cycle at 10% descending was required. Therefore, the enzyme loading in Cycle V was reduced from 50 FPU·$g^{-1}_{total\ solid}$ (Cycle I) to 30 FPU·$g^{-1}_{total\ solid}$, resulting in a 40% enzyme saving. By applying above enzyme loadings, similar sugar concentrations were produced for Cycles I to V (Fig. 4). The sugar concentrations in the first three cycles were slightly increased, and almost unchanged after Cycle III. Total sugar concentration in Cycle V (42.6 g·L^{-1}) was slightly reduced, whereas still higher than that of the Cycle I (41.3 g·L^{-1}). That may be caused by the increasing amount of straw solid accumulated after previous cycles, which hindered the mass transfer efficiency in the enzymatic hydrolysis. The results also indicated that 24 h may not be enough for thorough hydrolysis of RS, especially for the residual solids. Nevertheless, the sugar concentrations obtained from each five cycles were sufficient for further butanol fermentation (Fig. 4).

3.6. Biobutanol fermentation using $RS_{CombDA2}$ hydrolysates

The fermentability of $RS_{CombDA2}$ hydrolysates obtained from Cycle I (Hydrolysate I) and Cycle V (Hydrolysate V) was evaluated using butanol-producing strain *C. saccharobutylicum* DSM 13864. (Fig. 5). Control experiment was also carried out utilizing glucose instead of hydrolysates (Fig. 5A). The glucose concentration in control was set at the same level as total sugar of Hydrolysates I and V. The fermentation results were summarized in Table 3. Similar lagging phase and fermentation period were found by using glucose or hydrolysate medium. At 72 h, for glucose fermentation, the strain consumed 40.7 g·L^{-1} of

Table 3
Butanol fermentation from rice straw hydrolysates by *C. saccharobutylicum* DSM13864.

Carbon source	Total sugar		Butanol			Acetone-Butanol-Ethanol		
	Initial [g·L^{-1}]	Residual [g·L^{-1}]	Titer [g·L^{-1}]	Yield [g·g^{-1} total sugar]	Productivity [g·L^{-1}·h^{-1}]	Titer [g·L^{-1}]	Yield [g·g^{-1} total sugar]	Productivity [g·L^{-1}·h^{-1}]
Glucose	42.85	2.08	10.59	0.26	0.15	14.52	0.36	0.20
Hydrolysate from Cycle I	40.78	2.92	9.54	0.25	0.13	13.73	0.36	0.19
Hydrolysate from Cycle V	40.45	2.81	9.51	0.25	0.13	13.39	0.36	0.18

total sugar after 72 h, producing 10.6 g·L^{-1} of butanol, and the yield and productivity of butanol were 0.26 g·g$^{-1}_{\text{total sugar}}$ and 0.15 g·L^{-1}·h^{-1}. In the fermentation process of Hydrolysates I and V, 37.9 g·L^{-1} and 37.6 g·L^{-1} of total sugar were consumed, resulting in the same butanol yield and productivity of 0.25 g·g$^{-1}_{\text{total sugar}}$ and 0.13 g·L^{-1}·h^{-1}, respectively (Table 3). Therefore, similar butanol titers were achieved in glucose and hydrolysates fermentation, indicating that RS-Comb$_{DA2}$ hydrolysates prepared by the 5 th recycled cellulase could be used for fermentation without apparent inhibitory effect.

4. Conclusions

A novel dihydrogen-bonding DES, foraceline, was synthesized using choline chloride, formic acid and acetic acid at mole ratio of 1:1:1. Notable performance of was found with foraceline in the pretreatment of rice straw. A two-stage pretreatment of RS by combination of foraceline and Na_2CO_3 was more effective than that pretreated by single foraceline or Na_2CO_3. Here, the highest total sugar of 42.76 g·L^{-1} was reached. In the hydrolysis of RS$_{CombDA2}$ with cellulase recycle, sugar concentration in Cycle V was slightly increased than that of the first cycle, indicating the effectiveness of cellulase reutilization. For butanol fermentation by *C. saccharobutylicum* DSM 13864, butanol yield of 0.25 g·g$^{-1}_{\text{total sugar}}$ and productivity of 0.13 g·L^{-1}·h^{-1} were attained using Hydrolysates I (Cycle I) and V (Cycle V). Our results demonstrate that foraceline-Na_2CO_3 pretreatment method has excellent adaptability to the pretreatment of lignocellulosic biomass.

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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.cej.2017.09.176.

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