Acetone–butanol–ethanol production from corn stover pretreated by alkaline twin-screw extrusion pretreatment

Yuedong Zhang · Tongang Hou · Bin Li ·
Chao Liu · Xindong Mu · Haisong Wang

Received: 23 May 2013 / Accepted: 9 September 2013 / Published online: 10 October 2013
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Abstract In this study, the alkaline twin-screw extrusion pretreated corn stover was subjected to enzymatic hydrolysis after washing. The impact of solid loading and enzyme dose on enzymatic hydrolysis was investigated. It was found that 68.2 g/L of total fermentable sugar could be obtained after enzymatic hydrolysis with the solid loading of 10 %, while the highest sugar recovery of 91.07 % was achieved when the solid loading was 2 % with the cellulase dose of 24 FPU/g substrate. Subsequently, the hydrolyzate was fermented by *Clostridium acetobutylicum* ATCC 824. The acetone–butanol–ethanol (ABE) production of the hydrolyzate was compared with the glucose, xylose and simulated hydrolyzate medium which have the same reducing sugar concentration. It was shown that 7.1 g/L butanol and 11.2 g/L ABE could be produced after 72 h fermentation for the hydrolyzate obtained from enzymatic hydrolysis with 6 % solid loading. This is comparable to the glucose and simulated hydrozate medium, and the overall ABE yield could reach 0.112 g/g raw corn stover.

Keywords Alkaline twin-screw extrusion pretreatment · Enzymatic saccharification · Acetone–butanol–ethanol (ABE) · Corn stover · *Clostridium acetobutylicum* ATCC 824 · Fermentation

Introduction

Recently, great efforts have been dedicated to the second-generation biofuel research to make a more sustainable substitute for fossil-based energy. A variety of renewable resources, such as corn stover [1, 2], wheat straw [3] and wood [4], have been employed for the production of ethanol or butanol. Corn stover, which is an abundant and easily-collected agricultural waste, needs to be pretreated and enzymatically hydrolyzed into monosaccharides for the further use of microbes, as the microbes cannot efficiently and directly convert lignocellulosic biomass to ethanol or butanol due to its natural recalcitrance. A lot of methods for the preparation of fermentation culture from lignocellulosic biomass were reported, but the issues, such as the use of corrosive chemicals, potential environmental pollution, generation of inhibitory compounds, and high capital or operational costs limit their large-scale utilizations.

Extrusion pretreatment is a relatively novel physico-chemical method in which biomass is processed by means of heat, compression and shear forces, leading to physical disruption and chemical modifications of biomass during the passage through the extruder [5–9]. In our previous studies [10, 11], a novel pilot-scale alkaline twin-screw extrusion (ATSE) pretreatment process using the specially designed twin-extruder to pretreat corn stover was established. It was found that under relatively mild conditions (NaOH loading of 0.06 g/g biomass, biomass/liquid ratio of 1/2 (w/w), temperature of 99 °C, and 1 h heat preservation after extrusion), the ATSE pretreatment could effectively disrupt the lignin–carbohydrate complex and significantly improve the enzymatic digestibility of corn stover [11]. Compared with traditional pretreatment approaches like dilute acid/alkali pretreatment, steam explosion pretreatment (i.e., AFEX or ARP), the ATSE pretreatment exhibits several

Y. Zhang · T. Hou · B. Li · C. Liu · X. Mu (✉) ·
H. Wang (✉)

Key Laboratory of Biofuel, Qingdao Institute of Bioenergy
and Bioprocess Technology, Chinese Academy of Sciences,
Qingdao 266101, Shandong, China
e-mail: muxd@qibebt.ac.cn

H. Wang
e-mail: wanghs@qibebt.ac.cn

Y. Zhang
e-mail: zhangyd@qibebt.ac.cn

desirable characteristics: high sugar yield (about 80 %), no need of severe size-reduction prior to the extrusion, less water usage during extrusion, relatively low temperature (about 99 °C) and no extra heating needed [9–11].

Although pretreatment could improve the digestibility of biomass, some compounds (e.g., phenolic compounds, furfural, etc.) which are toxic to ABE fermentation can be generated during pretreatment process. Among the known inhibitory compounds, the phenolic compounds generated from lignin such as ferulic and coumaric acid derivatives have been proved to be stronger inhibitors than weak acids and furan derivatives [12, 13]. Therefore, several detoxification methods, including over-liming [14], peroxidase [15], activated charcoal and alkaline peroxide treatment [2], were applied to remove the inhibitory compounds. However, it has been recently reported that some soluble lignin compounds (SLC) could be released during enzymatic hydrolysis [2], and there is an improvement on ABE yield when the SLC is between 0.44 and 3.32 g/L, even though the fermentation was inhibited when SLC exceeded 1.77 g/L [2]. The improvement is associated with the unidentified compounds in SLC (released in enzymatic hydrolysis) [2], which are different from the phenolic compounds generated during pretreatment. Thus, controlling SLC of hydrolyzate in enzymatic hydrolysis is of great importance for the downstream production of ABE.

In this study, the ATSE pretreated corn stover was subjected to enzymatic hydrolysis after washing, and enzymatic hydrolysis of ATSE pretreated corn stover was optimized. In addition, the inhibitory compounds of the hydrolyzate and the subsequent acetone–butanol–ethanol (ABE) fermentation by *Clostridium acetobutylicum* ATCC 824 were investigated.

Materials and methods

Materials

Corn stover was harvested from Qingdao in Shandong Province (China) in October, 2011. Before pretreatment, the corn stover was cut into 2- to 5-cm pieces in length. The enzyme cocktail was obtained from KDN Biotech Co. Ltd. The activities of cellulase, xylanase and β -glucosidase were 60 FPU (filter paper unit)/mL, 760 IU (international unit)/mL and 30 IU/mL, respectively. Other chemicals (analytical grade) were bought from Sinopharm Chemical Reagent Co. Ltd. and used as received.

Pretreatment

The corn stover was pretreated by a pilot-scale ATSE machine manufactured by Tianzheng Co. (China) with a

capacity of 200 kg oven dry (o.d.) corn stover per hour [10, 11]. A NaOH solution and the biomass/liquid ratio of 1:2 were used in the ATSE pretreatment. The NaOH dosage was 8 wt. % based on the weight of corn stover (o.d.). The corn stover entered the extruder via a feed inlet continuously and was crushed by the twin-screw extruder. The screw speed was fixed at 325 rpm. Out of the ATSE machine, the samples were preserved in a thermal insulation tank for 1 h at about 99 °C.

The mechanism of ATSE pretreatment was discussed in our previous work [11]. In brief, the ATSE pretreatment is based on the use of a co-rotating twin-screw extruder as main treatment device. Figure 1 [11] shows the picture of the twin-screw and the basic operating principle of twin-screw extruder. The twin-screw extruder consists of two co-rotating intermeshing screws inside. Each screw consists of four transport screw elements (TSE) and four reversed screw elements (RSE). The biomass enters the main body of extruder via a feed inlet and then is pushed to the RSE by the action of TSE. RSE is the main screw element with threads whose pitch is opposite to the TSE. This results in accumulation and compression of the biomass fiber in the space between TSE and RSE as shown in Fig. 1. After crushed by transport force and the reversed force, generated by TSE and RSE respectively, the biomass is forced to pass through the skewed slots of the RSE. Then, the biomass enters the next section of TSE and RSE to be further squeezed and crushed. As repeated in this way, the biomass continuously passes through the twin-screw and thus is mechanically crushed. During the extrusion process, great mechanical forces (frictional force and shearing force) are generated among the biomass fibers and the screws. The high mechanical forces cause fibrillation and shortening of biomass fibers. In addition, during the extrusion process in ATSE machine, tremendous mechanical forces lead to the generation of a great amount of heat thereby significantly increasing the temperature (up to about 99 °C) of biomass. Therefore, without additional heat, the pumped alkali can be thoroughly mixed and reacted with the crushed biomass under proper conditions.

SEM results demonstrated the surface structure changes of corn stover before and after ATSE pretreatment (previously reported) [11]. After the ATSE pretreatment, the integrated structure of corn stover was broken into separated fibers and fiber bundles, and the fiber surface became coarse with fiber delamination. This kind of mechanical size reduction increases external/internal specific surface area, which benefits the enzymatic saccharification of biomass [5].

Washing process

The pretreated corn stover was washed with tap water four times through a 300-mesh nylon cloth until neutrality. For

Fig. 1 The picture of specially designed twin-screw extruder and the basic operating principle of twin-screw extruder. **a** The picture of the specially designed twin-screw extruder, **b** the schematic diagram of

the specially designed twin-screw extruder, **c** the sketch map of a pair of reversed screw element (RSE) and transport screw element (TSE)

each time, the ratio of washing water to the pretreated corn stover was 3:1 (w/w). The washed stock was then filtered through double-layered muslin cloth with the meshes of 300 and the solid residue was collected for enzymatic hydrolysis [16].

Enzymatic hydrolysis of the ATSE pretreated corn stover

For solid loading and enzyme dose tests, a mixture (20 mL) of pretreated corn stover (with varied solid loading from 2 to 10 %) and sodium citrate buffer (0.1 M, pH 4.8) was added in a 50-mL capped bottle. The solid loading for enzymatic hydrolysis tests is expressed as the weight of pretreated corn stover (o.d.) divided by the total weight of materials used (pretreated corn stover, enzymes and buffer) for hydrolysis. Enzyme cocktail was added in the bottle and the enzymatic hydrolysis was carried out in 50 °C with the agitation of 160 rpm for 48 h. Upon completion, the hydrolyzate was filtered to remove the sediment [17], and stored at 4 °C for the composition analyses and fermentation. Sugar recovery was defined as the percentage of total sugar in hydrolyzate divided by the total sugar in pretreated corn stover. For fermentation test, the enzymatic hydrolysis was carried out in a 1-L flask with 500 mL mixture under the optimized conditions.

Fermentation

Studies of solvent-producing fermentation were performed in 250 mL screw-capped bottles containing 100 mL medium. Nitrogen purging at the flow rate of 100 mL/min was conducted to generate anaerobic conditions for the fermentation. Before adding yeast extract and P2 stock [18] aseptically by filtration sterilization, all the hydrolyzate, simulate hydrolyzate and sugar medium were sterilized at 115 °C for 30 min, and all fermentation experiments were conducted at 37 °C with 90 rpm of stirring for 72 h.

Spores of *Clostridium acetobutylicum* ATCC 824 were maintained in distilled water at 4 °C. Before use, the spores (0.1 mL) were heat shocked at 75 °C for 2 min and transferred to reinforced clostridial media (RCM, Oxoid CM 149) for spore germination. The spores were rejuvenated anaerobically for 12 h, and then subcultured to a P2 medium containing 30 g/L glucose and incubated for another 6 h. Subsequently, the P2 medium was subcultured to the solvent-producing medium at a 5 % inoculation amount. The initial pH value of the subcultured medium was 5.5–5.7, and the final pH after fermentation was about 5.0 (the pH value was not controlled during fermentation). Samples taken from the bottles were centrifuged at 12,000 rpm for 2 min, and the supernatants were used for ABE fermentation and sugar analyses. All experiments

Table 1 Characterization of feedstock and pretreated corn stover

	Dry weight (tone)	Component ^a (%)							
		Glucan	Xylan	Arabinan	Acetyl	Acid soluble lignin	Acid insoluble lignin	Extractive	Ash
Component content of raw corn stover ^a	0.450 ^b	32.31 ± 0.23	16.78 ± 0.14	1.94 ± 0.06	2.98 ± 0.08	1.05 ± 0.09	21.98 ± 1.25	18.45 ± 0.76	7.44 ± 0.20
Component content of pretreated stover ^a	0.268 ^c	53.87 ± 0.65	20.19 ± 0.25	1.29 ± 0.10	0.36 ± 0.02	0.45 ± 0.05	8.55 ± 0.78	–	–
Recovery rate (%) ^d		99.3	72.2	39.6	12.1	25.5	23.2		

^a Oven dry weight basis

^b Moisture content of raw corn stover: 10.75 %

^c Moisture content of pretreated corn stover after washing: 76.94 %

^d Recovery rate (%) = $\frac{\text{dry weight of pretreated stover} \times \text{component content of pretreated stover}}{\text{dry weight of raw stover} \times \text{component content of raw stover}} \times 100$

were carried out at least twice to ensure reproducibility and the average was reported.

Analytical methods

All biomass composition analyses were conducted in triplicate following NREL analytical procedure [19]. The analyses of ABE, sugar and acid content in fermentation broth were conducted as mentioned in our previous work [10, 11]. SLC were identified by ultraviolet spectrophotometry and the SLC concentration was calculated according to the following equation [20]:

$$C_{\text{SLC}} = 4.187 \times 10^{-2} (A_{280} - 3.19 \times U)$$

where C_{SLC} is the SLC concentration (g/L); A_{280} is the absorbance reading at 280 nm after the correction for dilution; U is the enzymatic activity of the sample (FPU/mL). In the equation, the interface from enzyme absorbance has been removed. The sodium citrate buffer was used as a blank test. The 3,5-dinitrosalicylic acid method was used for quick analysis of total reducing sugar.

Results and discussion

Characterization of corn stover before and after ATSE pretreatment

The component analyses of feedstock and pretreated corn stover are shown in Table 1. It is seen that, after ATSE pretreatment, the content of glucan and xylan increased from 32.31 and 16.78 % (raw material) to 53.87 and 20.19 %, respectively, and 99.3 % of the glucan and

Fig. 2 The impact of solid loading of enzymatic hydrolysis on sugar recovery in different enzyme dose

72.2 % of xylan were preserved. Table 1 also presents that the content of acid insoluble lignin decreased from 21.98 to 8.55 %. The significant lignin removal (77 %) is mainly due to the severe disruption of corn stover during physical extrusion [10, 11], thus leading to a better chemical modification of biomass via facilitated the penetration of NaOH and delignification reactions [9].

Enzymatic hydrolysis of ATSE pretreated corn stover

Figures 2 and 3 present the impact of solid loading and cellulase dose of enzymatic hydrolysis on sugar recovery and sugar concentration, respectively. As shown in Fig. 3, the highest sugar concentration of 68.17 g/L was achieved at 10 % solid loading with the cellulase dose of 24 FPU/g,

Fig. 3 The impact of solid loading of enzymatic hydrolysis on reducing sugar concentration in different enzyme doses

and the corresponding sugar recovery was 80.9 % (Fig. 2). However, the highest sugar recovery of 91.07 % (Fig. 2) was achieved at the 2 % solid loading with the cellulase dose of 24 FPU/g. Figure 2 also exhibited that the sugar recovery was significantly affected by enzyme dose. For the case of 2 % solid loading, when cellulase dose increased from 6 to 12 FPU/g, the sugar recovery improved about 11 %, while when cellulase dose elevated from 18 to 24 FPU/g, the sugar recovery just increased about 3 %. Similar trends can also be found for high solid loading hydrolysis. This is mainly due to the difficult mass transition (enzyme penetration), because a high sugar recovery could be achieved with a high solid loading for enzymatic hydrolysis, as long as the mass transition is good enough [21]. In addition, as the ATSE pretreatment was alkali combined, some of the known microbial inhibitors produced in the acid pretreatment [e.g., furfural, hydroxymethyl furfural (HMF)] were not detected in spent liquor of washing. However, after 48 h hydrolysis, 11.6–12.3 % of lignin was dissolved in the hydrolyzate (Fig. 4). This indicates that SLC could be generated during the enzymatic hydrolysis of lignocelluloses, which was also reported by Wang et al. [2] and Alvira et al. [22]. The SLC could be some small molecular lignin which generated during the alkali pretreatment. This small part of lignin re-deposited to the hemicellulose or cellulose after pretreatment [25] and was not removed in washing process. During enzymatic hydrolysis of pretreated corn stover, the re-deposited small molecular lignin was released when the hemicellulose and cellulose were hydrolyzed by enzymes, and thus dissolved in the hydrolyzate. Furthermore, the enzyme cocktail was made of the crude enzymes which were produced by lignocellulose-degrading microbes, and it contains a little laccase which could degrade some lignin

Fig. 4 SLC in the hydrolyzate of different solid loading Lignin content is 9.00 % (w/w) in the pretreated corn stover. The enzyme dosage was 18 FPU/g substrate

remained in substrate after pretreatment and washing, thus releasing a little SLC during enzymatic hydrolysis. For higher sugar utilization, no additional detoxification which could cause sugar loss was applied to the hydrolyzate. Because the fermentation could be improved when SLC is below 0.89 g/L [2], the hydrolyzate obtained via enzymatic hydrolysis with 6 % solid loading was used for the fermentation study, and the corresponding SLC was 0.74 g/L in 500-mL scale.

ABE fermentation

ATCC 824 was one of the most studied solvent-producing bacteria [2, 23, 24], and it can be used to ferment glucose, xylose, arabinose and cellobiose. The highest ABE concentration produced by ATCC 824 was 18.7 g/L in a 60-g/L glucose P2 medium, as reported in previous research [23]. To investigate the hydrolyzate sugar utility by ATCC 824, three comparative experiments were carried out using a simulated hydrolyzate medium which has a similar mixed sugar concentration to the actual hydrolyzate, a glucose medium and a xylose medium which have a similar total sugar concentration to the actual hydrolyzate. The contents of the four mediums can be seen in Table 2. The concentrations of glucose, xylose and arabinose during ABE fermentation are shown in Fig. 5. ATCC 824 utilized the xylose like glucose when there was no glucose in the culture, as presented in Figs. 4d and 5c. The glucose was utilized faster than xylose in hydrolyzate and mixed sugar medium and exhausted first in the two mediums. At the end of fermentation, there were 0.5 g/L total sugars left in the simulated hydrolyzate medium (Fig. 5b) and 3.1 g/L total sugars left in the hydrolyzate (Fig. 5a). The ABE

Table 2 Contents of solvent-producing mediums

	Cellobiose (g/L)	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Total sugar (g/L)	P2 stock %(v/v)	Yeast extract (g/L)
Hydrolyzate	2.7	26.7	11.6	0.8	42.4	1	1
Simulated hydrolyzate medium	2.7	26.7	11.6	0.8	42.4	1	1
Glucose medium		42.4	–	–	42.4	1	1
Xylose medium		–	42.4	–	42.4	1	1

Fig. 5 Sugar utilization in different mediums. **a** Hydrolyzate, **b** simulated hydrolyzate medium, **c** glucose medium, **d** xylose medium

concentrations as functions of fermentation time in the four different mediums are shown in Fig. 6. As can be seen, after 72 h fermentation, butanol concentrations of 7.1, 7.0, 7.0 and 7.7 g/L can be achieved in hydrolyzate, simulated hydrolyzate medium, glucose medium and xylose medium, respectively, and the corresponding total ABE concentration were 11.2, 11.1, 11.5 and 12.4 g/L, respectively (Fig. 7). The ABE yield from total sugar utilized in all medium ranged from 0.26 to 0.28 g/g sugar, as shown in Fig. 8. However, when SLC was as low as 0.74 g/L, the

sugar utilization was 92.7 % which was still inhibited, compared to the simulated hydrolyzate (98.8 %) and glucose medium (100 %). But the yield of ABE (0.286 g/g sugar) was higher than the simulated hydrolyzate (0.264 g/g sugar) and glucose medium (0.273 g/g sugar). Wang et al. [2] reported that there was an improvement on ABE yield when the SLC is between 0.44 and 3.32 g/L while the utilization of sugar was inhibited when SLC exceeded 1.77 g/L. Thus, the concentration of ABE was higher than the control medium when the SLC was between 0.44 and

Fig. 6 ABE fermentation of different media. **a** Hydrolyzate, **b** simulated hydrolyzate medium, **c** glucose medium, **d** xylose medium

Fig. 7 The final ABE concentration in different media

Fig. 8 Efficiency of fermentation in different media

0.89 %. In our case, although the concentration of ABE was similar, the sugar utilization and ABE yield of hydrolyzate had an obviously difference from the control medium. The ABE yield was improved like the Ref. [2], but the utilization was inhibited when the SLC was as low as 0.74 g/L. The different behavior is possibly due to the different composition/structure of SLC derived from different pretreatment methods. Reference [2] used alkaline peroxide pretreatment which could make the phenolic groups of the lignin decomposed, and hence the SLC generated during enzyme hydrolysis could have less phenolic groups compared to the ATSE pretreatment. More phenolic groups in SLC may lead to the different behavior of fermentation samples, but more specific studies (e.g., quantitative analysis of the composition of SLC) are needed in future. All in all, 0.112 g/g raw corn stover (o.d.) of ABE yield can be achieved, indicating that hydrolyzate obtained from enzymatic hydrolysis of ATSE pretreated corn stover after washing could be a good substrate for ABE production.

Conclusion

A high sugar recovery was obtained at high solid loading (6 %) enzymatic hydrolysis using the ATSE pretreated corn stover after washing. The enzymatic hydrolyzate with low SLC concentration of 0.74 g/L obtained from the enzymatic hydrolysis was chosen for ABE fermentation test. The reducing sugar concentration of 42.4 g/L and sugar recovery of 83.8 % were achieved after hydrolysis. In addition, without any additional detoxification process, about 70 % of the fermentable sugar in the corn stover was utilized by the microbe, the hydrolyzate had similar fermentation performance to the controlled glucose and simulated hydrolyzate medium in ABE concentration, and the yield of ABE was 0.112 g/g raw corn stover.

Acknowledgments We are grateful for the support of the Natural Science Foundation of China and Shandong Province (No. 21206814, No. 21201174 and BS2009ZZ001), the SHELL Research Foundation as well as the National High Technology Research and Development Program (“863” program) of China (No. 2012AA022301).

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