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RESEARCH PAPER

Improving cellulosic ethanol fermentation efficiency by converting endogenous water-soluble carbohydrates into citric acid before pretreatment

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Abstract
Water-soluble carbohydrates in raw lignocellulosic biomass are converted into hydroxymethylfurfural (HMF) in the acid-based pretreatment, thus increasing the detoxification intensity and reducing the fermentation efficiency of cellulosic ethanol. Therefore, reducing water-soluble carbohydrates in raw corn stover is crucially important to reduce the inhibitors' generation and improve the ethanol fermentation efficiency. In this study, aerobic solid-state fermentation of corn stover by inoculating *Aspergillus niger* spores converted 83% of the endogenous water-soluble carbohydrates into citric acid, leading to the decrease of 41% of HMF generation and 8% of sulfuric acid usage during the dry acid pretreatment. The reduced inhibitor generation improved the ethanol fermentability by 11% more ethanol than that of the corn stover without water-soluble carbohydrates' removal. This suggests that the removal of the water-soluble carbohydrates before pretreatment significantly reduced the inhibitors' generation in pretreatment and improved the fermentation efficiency of cellulosic ethanol.

Keywords Water-soluble carbohydrates · Solid-state citric acid fermentation · Hydroxymethylfurfural · Corn stover · Cellulosic ethanol

Introduction

The biofuels or bio-based chemical from lignocellulose biomass is produced by multiple operation steps of pretreatment, enzyme hydrolysis, fermentation, and recovery. Beside cellulose and xylan, lignocellulose contains certain amount of endogenous water-soluble carbohydrates (WSC) including glucose, fructose, or sucrose [1]. The content of water-soluble carbohydrates in corn stover accounts for 4–12% by dry weight percentage and varies with growth regions, irrigation, soil, harvest operation, transportation, and storage [1–3]. The water-soluble carbohydrates are easily converted to 5-hydroxymethylfurfural (HMF) at high temperature and low pH conditions of pretreatment [4]. HMF inhibits the cell growth and metabolism of fermenting strains in the consequent fermentation step. Therefore, the removal of water-soluble carbohydrates from lignocellulose biomass before pretreatment could reduce HMF generation and improve fermentation efficiency. Up to now, few reports were found on the removal of water-soluble carbohydrates and its effect on cellulosic ethanol fermentation.

In this study, the water-soluble carbohydrates in raw corn stover were converted into citric acid by aerobic solid-state fermentation by inoculating *Aspergillus niger* spores before pretreatment. The evaluation was compared using the raw corn stover as a control under the same pretreament, biodetoxification and simultaneous saccharification and co-fermentaiton (SSCF) conditions. The water-soluble carbohydrates' reduction and the citric acid generation reduced the usage of sulfuric acid and HMF formation in the consequent pretreatment. This is beneficial to improve the subsequent biodetoxification efficiency and cellulose ethanol fermentation efficiency.

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Materials and methods

Raw materials and reagents

Corn stover was harvested from Tongliao, Inner Mongolia, China in fall 2016, containing 33.2% of cellulose, 24.9% of hemicellulose, 20.8% of lignin, and 6.2% of ash (based on dry weight base, w/w) determined by NREL protocols [5, 6]. Water-soluble carbohydrates in corn stover were measured by extracting 4 g of dry corn stover with 80 mL distilled water and shaking vigorously in a 250 mL flask at 30 °C for 2 h [4]. Cellulase enzyme Cellic CTec 2.0 was purchased from Novozymes (China), Beijing, China. The filter paper activity was determined to be 203.2 FPU/mL, 4900 CBU/mL, and 87.3 mg/mL [7–9].

Strains and medium

Aspergillus niger SIIM M288 was obtained from Shanghai Industrial Institute of Microbiology (SIIM), Shanghai, China and cultured in the medium containing 70 g glucose/L; 2.5 g NH_4Cl/L; 2.5 g KH_2PO_4/L; 0.25 g $MgSO_4 \cdot 7H_2O$/L; 2.36×10^{-4} g $CuSO_4 \cdot 5H_2O$/L; 1.1×10^{-3} g $ZnSO_4 \cdot 7H_2O$/L; 6.45×10^{-3} g $FeSO_4 \cdot 7H_2O$/L; and 3.6×10^{-3} g $MnCl_2 \cdot 4H_2O$/L.

Amorphotheca resinae ZN1 was isolated in our previous work and stored in China General Microorganism Collection Center (CGMCC) with the registration number 7452 [10].

Saccharomyces cerevisiae XH7 was an engineered strain and cultured in YPD medium containing 20 g glucose/L, 20 g peptone/L, and 10 g yeast extract/L or corn stover slurry for activation and adaption procedure [11].

Solid-state citric acid fermentation

Solid-state citric acid fermentation was conducted by inoculating *A. niger* SIIM M288 onto corn stover [33% (w/w) of water, 28 °C and the initial pH of 5.2] for 10 days in sealed plastic bags. Each bag contained about 650 g of corn stover (wet weight) and the samples were periodically withdrawn for analysis of glucose, xylose, citric acid, and pH value. The aerobic solid-state fermentation for removing the water-soluble carbohydrates in raw lignocellulose can be carried out by spraying *Aspergillus niger* spores and fresh water during the feedstock storage period in future large-scale production.

Pretreatment, biodetoxification, and SSCF

Corn stover was dry-acid pretreated using 3.5–3.8 g sulfuric acid per 100 g dry feedstock at a solid/liquid ratio of 3:2 (w/w) and 175 °C for 5 min using the base pH approaching method [11, 12]. The base pH method contained two simple steps in flasks. At first, the 2 g dry corn stover is mixed with 100 mL deionized water and shook vigorously at 30 °C for 30 min. The pH value of the suspension slurry is measured as the "base pH value". The second step is to adjust the sulfuric acid usage by titration of the feedstock slurry to the base pH value. The pretreatment efficiency was assayed by measuring the enzymatic hydrolysis yield of cellulose according to the NREL LAP-009 protocol [13]. Briefly, 2.5% (w/w) of the pretreated feedstock solids was hydrolyzed using 20 FPU/g DM at pH 4.8, 50 °C for 72 h. The experiments were carried out in duplicate.

The pretreated corn stover was biodetoxified to remove the inhibitor compound [10, 14]. Briefly, the feedstocks were neutralized using 20% (w/w) $Ca(OH)_2$ suspension to pH 5–6. *A. resinae* ZN1 was inoculated at 10% (w/w), 28 °C for 30 h in a 15 L bioreactor with 0.8 vvm of aeration (the ratio of the air volumetric flow rate in liters per minute to the feedstock volume in liters).

Cellulosic ethanol fermentation was conducted in simultaneous saccharification and co-fermentaiton (SSCF) using the pretreated and biodetoxified corn stover at pH 5.5 in a 5L helical agitated bioreactor. After 12 h pre-hydrolysis at 50 °C, *S. cerevisiae* XH7 was inoculated and the SSCF was at 30 °C for 108 h. The nutrients added included 2 g KH_2PO_4/L, 2 g $(NH_4)_2SO_4$/L, 1 g $MgSO_4$/L, 10 g yeast extract/L.

Analysis of sugars, ethanol, citric acid, and inhibitors

Glucose, fructose, xylose, ethanol, citric acid, acetic acid, furfural, and HMF were measured using HPLC (LC-20AD pump, RID-10A detector, Shimadzu, Kyoto, Japan) with a Bio-Rad Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 65 °C and 0.6 mL/min of 5 mM H_2SO_4 solution as the mobile phase.

Calculation of ethanol yield

Ethanol yield was calculated according to the modified method by taking into account the ethanol formation and the water loss in the hydrolysis [15].

Results and discussion

Conversion of water-soluble carbohydrates into citric acid and pretreatment performance

The water-soluble carbohydrates included 32.8 mg of glucose and 30.2 mg of fructose per gram of dry corn stover, according to the determination method of water extraction.

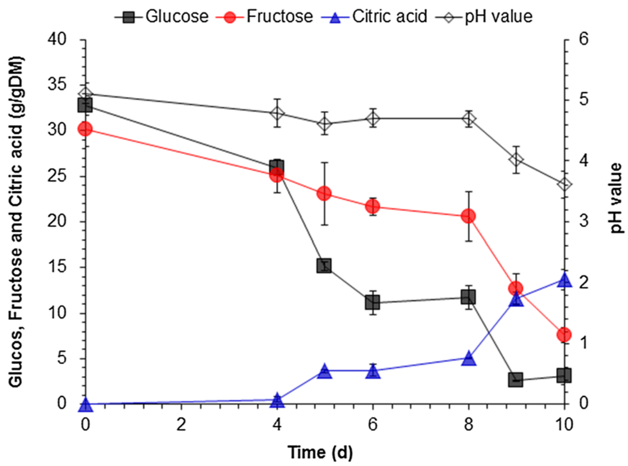

Fig. 1 The formation of citric acid from water-soluble carbohydrates in corn stover by aerobic solid-state fermentation. Without exogenous nutrients, the fermentation was conducted at 28 °C, and the initial pH 5.2 for 10 days in sealed plastic bags by *A. niger* SIIM M288. The experiments were carried out in duplicate, and the data used here were taken from the average of two parallel experiments

The corn stover was inoculated with *A. niger* M288 spores and fermented for 10 days according to the procedure in Section "Solid-state citric acid fermentation". Figure 1 shows that the 83% of the water-soluble carbohydrates was consumed, converting 91% of glucose and 75% of fructose, respectively, to generate 13.7 mg of citric acid per gram of dry corn stover. And the pH value of corn stover was reduced to 3.6 from 5.2.

Consequently, the corn stover feedstocks with and without the removal of water-soluble carbohydrates were dry-acid pretreated. The sulfuric acid usage for the pretreatment was reduced from 38 to 35 mg/g due the citric acid produced partially compensating the acid catalyst as calculated by the base pH approaching method [12]. After the pretreatment, the two corn stover feedstocks contained almost the same cellulose (33.3 ± 0.1% vs. 34.5 ± 1.22%), hemicellulose (2.9 ± 0.14% vs. 2.9 ± 0.31%), xylan oligomers (26.3 ± 2.3 vs. 25.4 ± 3.3 mg/g dry matter), and the similar enzymatic hydrolysis yield (96.0 ± 0.1% vs. 94.3 ± 2.1%). The HMF generation was significantly reduced by 41% (6.2 ± 0.1 mg vs. 10.5 ± 0.2 mg per gram dry corn stover) (Fig. 2). The result indicates that the aerobic solid-state fermentation effectively removed the water-soluble carbohydrates in corn stover, and significantly reduced the HMF generation during pretreatment operation.

Ethanol fermentation using corn stover feedstock after water-soluble carbohydrates' removal

The pretreated corn stover feedstocks with and without water-soluble carbohydrates removal were biodetoxified to remove the inhibitors generated from pretreatment operation. Figure 2 shows that furfural was completely removed after 30 h. In this operation, citric acid produced by aerobic solid-state fermentation reacts with calcium hydroxide to produce insoluble calcium citrate. For the raw corn stover, 71% of HMF and 10% of acetic acid were degraded by *A.*

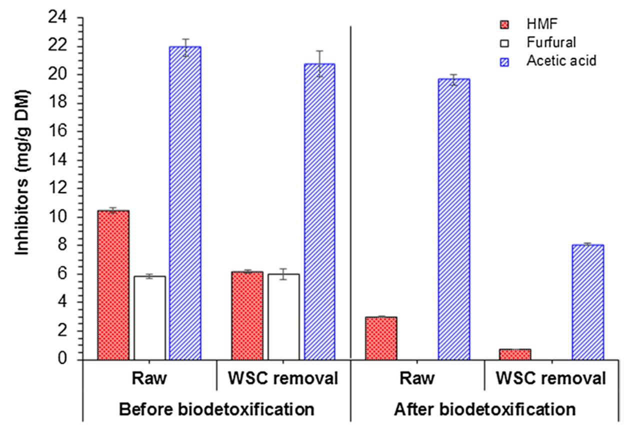

Fig. 2 Degrading of inhibitors in the pretreated corn stover feedstocks by biodetoxification. The biodetoxification assays were conducted using the two pretreated corn stover (with and without the removal of water-soluble carbohydrates) at 28 °C and 0.8 vvm (the ratio of ventilatory capacity to volume of the feedstocks in 1 min) for 30 h by *A. resinae* ZN1 in a 15-L bioreactor. The experiments were carried out in duplicate, and the data used here were taken from the average of two parallel experiments. *WSC* water-soluble carbohydrates, *HMF* 5-hydroxymethylfurfural, *DM* dry matter

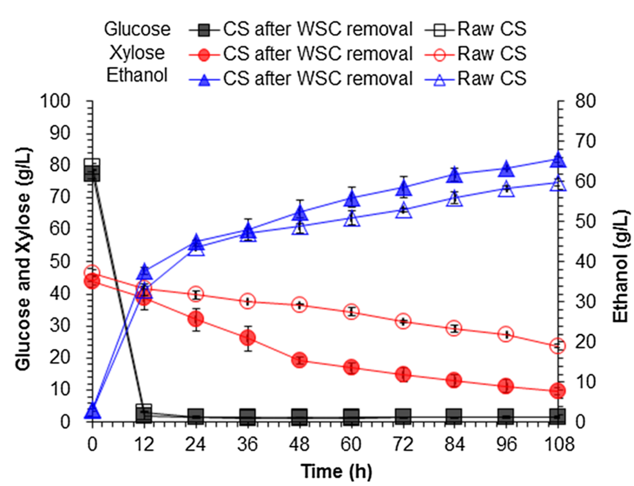

Fig. 3 Ethanol fermentability evaluation of the two pretreated and biodetoxified corn stover (with and without the removal of water-soluble carbohydrates) by SSCF at the same experimental conditions. The SSCF was conducted at 30% (w/w) of solids loading, 10 mg total protein/g cellulose, 50 °C, and pH 4.8 for 12 h in the prehydrolysis stage and 30 °C and pH 5.5 for 108 h by *S. cerevisiae* XH7 in the SSCF stage. The experiments were carried out in duplicate, and the data used here were taken from the average of two parallel experiments. *CS* corn stover, *WSC* water-soluble carbohydrates

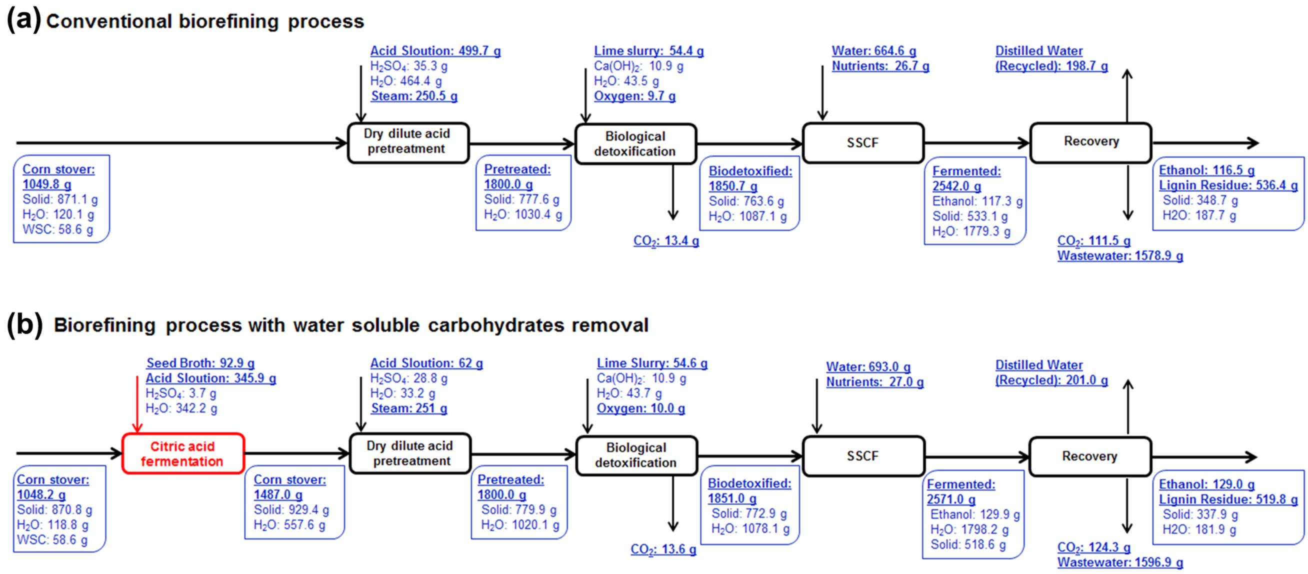

Fig. 4 Mass balance analyses for cellulosic ethanol production from two pretreated and biodetoxified corn stover (with and without the removal of water-soluble carbohydrates) in the present biorefining chain starting from the raw corn stover. **a** Conventional biorefining process; **b** biorefining process with water-soluble carbohydrates' removal. The mass balance of biorefining process referred to the method [16]

resinae ZN1, leaving 3.04 mg/g dry matter of HMF and 19.65 mg/g dry matter of acetic acid. For the corn stover after the removal of water-soluble carbohydrates, 88% of HMF and 61% of acetic acid were degraded and leaving 0.74 mg/g dry matter of HMF and 8.10 mg/g dry matter of acetic acid. The result indicates that the biodetoxification efficiency was significantly enhanced by the removal of water-soluble carbohydrates before pretreatment.

The simultaneous saccharification and co-fermentation (SSCF) of the two pretreated and biodetoxified corn stover feedstocks were performed under 30% (w/w) solids loading (Fig. 3). The ethanol titer and yield were a clear improvement: 65.6 g ethanol/L obtained from the water-soluble carbohydrates removal corn stover, while only 59.9 g ethanol/L was obtained from the raw corn stover. Concerning xylose conversion, 49.0% of xylose was converted into ethanol when the raw corn stover was used, while 78.0% of xylose was converted to ethanol when using the water-soluble carbohydrates' removal corn stover. The reduced HMF and acetic acid concentrations in the water-soluble carbohydrates removal corn stover contributed to the increased ethanol yield and xylose conversion ratio.

Figure 4 shows the mass balance of dry corn stover in the present biorefining chain starting from the raw corn stover, in which no wastewater was generated in the core steps of pretreatment, detoxification, fermentation until the separation. The ethanol product from the water-soluble carbohydrates' removal corn stover was higher around 11% than that of the raw corn stover.

Conclusion

The water-soluble carbohydrates are easily converted to 5-hydroxymethylfurfural (HMF) at high temperature and low pH conditions of pretreatment. The aerobic solid-state fermentation before pretreatment converted 91% of glucose and 75% of fructose, and generated 13.7 mg of citric acid per gram of dry corn stover. Under the same pretreatment efficiency, the HMF generation in the pretreatment was significantly reduced by 41% and the ethanol fermentation efficiency was improved. The results provided an effective way to reduce the HMF formation in pretreatment and improve ethanol fermentation efficiency by the removal of water-soluble carbohydrates in raw lignocellulose biomass.

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Compliance with ethical standards

Conflict of interest Authors do not have any conflicts of interest.

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