Biomass Conversion and Biorefinery
https://doi.org/10.1007/s13399-020-01183-1

ORIGINAL ARTICLE

Upgrading steam pretreatment by converting water-soluble carbohydrates into lactic acid prior to pretreatment

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Received: 14 July 2020 / Revised: 21 November 2020 / Accepted: 1 December 2020

Abstract
Steam pretreatment is an effective approach to open up the intact lignocellulosic matrix for biofuels and animal feed production. However, crop residues especially corn stover contain certain amounts of water-soluble carbohydrates (WSC), which are easily degraded into toxic 5-hydroxymethylfurfural (HMF) at high temperature and bring negative effects on subsequent utilization of lignocellulose biomass. Few studies have focused on the WSC loss and proposed a corresponding solution to utilize them in steam pretreatment. This study introduced a solid-state fermentation prior to steam pretreatment of corn stover to convert WSC into lactic acid, which could catalyze the hydrolysis of hemicellulose and acetyl groups in pretreatment. Compared to the regular steam pretreatment at the same severity (180 °C for 5 min), the conversion yields of xylan and arabinan in the steam pretreatment were both increased from 9.6% and 37.2% to 28.8% and 69.6%, respectively. Cellulose conversion also increased from 48.1% to 50.7% in the subsequent enzymatic hydrolysis. On the other hand, the generation of the toxic HMF reduced by 42.1% due to the pre-conversion of WSC into the relatively thermal stable lactic acid. This study provided an effective approach to process corn stover with higher hydrolysis yield and lower inhibitor generation.

Keywords Lignocellulose · Steam pretreatment · Water-soluble carbohydrates (WSC) · 5-Hydroxymethylfurfural (HMF) · Enzymatic hydrolysis

1 Introduction

Biorecalcitrance of lignocellulose biomass restricts its utilization to produce biofuels, bio-based chemicals, and ruminant feed diets [1, 2]. Pretreatment is the key step to overcome the innate biomass recalcitrance and render the biomass more accessible to cellulase by altering its chemical compositions or physical structures. Several pretreatment approaches have been applied in processing lignocellulose biomass, such as mechanical pretreatment, alkali-based pretreatment (sodium hydroxide, ammonia fiber expansion), acid-based pretreatment (steam, dilute acid), organosolv pretreatment, ionic liquid pretreatment, and biological pretreatment [3–9]. Steam pretreatment is widely utilized to process corn stover biomass in biofuels and ruminant feed plants because of its potential for easy hydrolysis of the hemicelluloses. It acts as an excellent environment-friendly method due to high efficiency, unnecessary addition of chemicals or solvent, lower capital investment, possibility of using high chip size, and feasibility at industrial-scale applications [3–5, 9–12].

Crop residues such as corn stover, wheat straw, and rice straw generally contain certain amounts of water-soluble carbohydrates (WSC) including glucose, fructose, and sucrose [13]. In corn stover, WSC accounts for 4%-12% dry weight of the biomass depending on the maize hybrids, cultivation, growth period, harvest operation, and storage conditions [13–17]. These sugars are easily degraded into 5-hydroxymethylfurfural (HMF) at high temperature [18], and it will bring negative effects on subsequent enzymatic hydrolysis and microbial conversion [19–21].

Our previous study has confirmed that bioconversion of WSC before pretreatment could significantly reduce the HMF generation in dilute sulfuric acid pretreatment and consequently facilitate cellulosic ethanol production [20]. In the other work, we found that the generated lactic acid from WSC maintained stable in dilute sulfuric acid pretreatment and accumulated to the final cellulosic lactic acid production [21].

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Published online: 20 January 2021

However, dilute acid pretreatment with sulfuric acid catalyst was too harsh for a few scenarios such as ruminant feed diet production. It is necessary to expand this method to upgrade the common used mild steam pretreatment. Here we hypothesized that the generated lactic acid catalyst could improve pretreatment efficiency and facilitate subsequent enzymatic hydrolysis. Also, this technology would reduce toxic HMF formation with the pre-conversion of WSC.

In this study, corn stover was fermented at solid state to convert WSC into lactic acid prior to steam pretreatment. Chemical composition analysis and enzymatic hydrolysis were conducted to show the benefits of lactic acid-assisted steam pretreatment. This study provided an effective approach to process corn stover with higher hydrolysis yield and lower inhibitor generation.

2 Materials and methods

2.1 Raw materials

Mature corn stover was harvested from Tongliao, Inner Mongolia, China in the fall of 2016. The harvested corn stover was air dried for over 2 weeks and ground using a hammer crusher to pass it through the 10-mm (diameter) apertures, and then it was sealed in plastic bags and stored at room temperature until use. The corn stover contained 32.4% of cellulose, 22.4% of xylan, 3.8% of arabinan, 20.8% of lignin, and 6.2% of ash by weight percentage measured according to NREL protocols [22]. Water-soluble carbohydrates (WSC) in corn stover were extracted by mixing 5 g of dry corn stover with 100 mL distilled water and shaking vigorously in a 250-mL flask at 30 °C for 1 h [20, 21], and determined to be 24.8 mg/g of glucose and 32.0 mg/g of fructose on a dry basis of corn stover. Other WSC were negligible in this study due to their low content in this batch of corn stover.

2.2 Strains and culture

The lactic acid bacteria *Pediococcus acidilactici* TY112 was stored in China General Microorganisms Collection Center (CGMCC), Beijing, China with the registration number of 8664 [23]. The MRS medium for seed culture contained 20 g/L of glucose, 10 g/L of yeast extract, 10 g/L of peptone, 2 g/L of diammonium hydrogen citrate, 5 g/L of sodium acetate, 0.3 g/L of $MgSO_4$, 2 g/L of K_2HPO_4, and 0.23 g/L of $MnSO_4$. One vial of *P. acidilactici* TY112 stock was inoculated into the 20-mL seeding medium and cultured at 42 °C for 12 h, and then 10% (v/v) of the culture was inoculated into the fresh seed medium at 42 °C for 5 h before the final inoculation into raw corn stover [21, 23, 24].

2.3 Enzymes and reagents

Commercial cellulase Cellic CTec 2.0 was purchased from Novozymes, Beijing, China. The filter paper activity was 203.2 FPU/mL determined using the NREL protocol LAP-006 [25], and the cellobiase activity was 4900 CBU/mL using the method of Ghose [26]. The protein concentration was 87.3 mg/mL determined by the Bradford method [27].

2.4 Solid-state fermentation

Corn stover feedstock was mixed with fresh water at the solid/liquid ratio of 2:1 (w/w), and then seed broth of *P. acidilactici* TY112 was sprayed on the feedstock at the weight ratio of 1% (w/w, based on the wet matter). The inoculated corn stover was squeezed and filled into airtight plastic bags (15 kg wet materials), and then stored at 25 °C for 7 days [21].

Samples were withdrawn and mixed with deionized water into slurries at the solid-liquid ratio of 1:20 and shaken vigorously in a 250-mL flask at 30 °C for 1 h [21]. The pH value of the slurry was measured by a pH sensor, and the supernatant of the slurry was collected by centrifugation at 11,167*g* for 5 min and used for analysis of lactic acid and sugar content [6, 21, 23].

2.5 Steam pretreatment

Steam pretreatment was conducted in a 20-L reactor with a single helical ribbon stirrer [28]. The saturated vapor at 1.6 MPa and 201 °C was jetted onto the corn stover feedstock with mild agitation. The temperature was controlled in the range of 120-180 °C by the adjustment of the steam injection rate. The initial solid/liquid ratio was 2:1 by mixing the feedstock with fresh water, and the solid content after pretreatment was around 50% (w/w).

2.6 Hydrolysis assay

The enzymatic hydrolysis was carried out according to the NREL protocol LAP-009 [29]. A total of 0.5 g of corn stover (dry matter), 10 mL of 0.1 M citrate buffer, and 10 mL of deionized water were added into a 100-mL flask to make the 2.5% (w/w) solids loading slurry and pH was adjusted to 6.0 by 5 M NaOH solution. A total of 80 μL tetracycline (10 mg/mL in 70% ethanol) and 60 μL cycloheximide (10 mg/mL in deionized water) were added to avoid the microbial contamination. The cellulase loading was 20 FPU/g DM and the hydrolysis was lasted for 48 h at 39 °C and 150 rpm in a water-bath shaker. The cellulose conversion was calculated according to the formula [29]:

$$\text{Cellulose conversion } (\%) = \frac{(C_{glu}\text{-}C_0) \times V}{m \times f\text{cellulose} \times 1.111 + \frac{m \times C\text{oliglu}}{1000}} \times 100\%$$

where C_{glu} is the final glucose concentration after 48 h hydrolysis (g/L), C_0 is the initial glucose concentration in the substrate and enzyme blank (g/L), V is the volume of the total liquid in the hydrolysis process (L), m is the weight of added dry corn stover (g), $f_{cellulose}$ is the cellulose content of corn stover (%), and C_{oliglu} is the glucooligomer content of corn stover (mg/g DM).

2.7 Analysis of sugars, L-lactic acid, and inhibitors

Polysaccharides were analyzed using a two-step sulfuric acid hydrolysis method [22]. Oligosaccharides were measured by a one-step sulfuric acid hydrolysis method [30]. Sugars were analyzed by HPLC (LC-20AD pump, RID-10A refractive index detector, Shimadzu, Kyoto, Japan) with a HPX-87P column (Bio-rad, Hercules, CA, USA) at 80 °C and 0.6 mL/min of sterilized deionized water. Lactic acid, acetic acid, HMF, and furfural were analyzed using HPLC (LC-20AD pump, RID-10A refractive index detector, Shimadzu, Kyoto, Japan) with a HPX-87H column (Bio-Rad, Hercules, CA, USA) at 65 °C and 0.6 mL/min of 5 mM H_2SO_4.

Phenolic compounds including vanillin, syringaldehyde, and 4-hydroxybenzaldehyde were analyzed using HPLC (LC-20AT pump, UV/VIS detector SPD-20A, Shimadzu, Kyoto, Japan) with a YMC-Pack ODS-A column (YMC, Tokyo, Japan) operated at 35 °C, flow rate of 1.0 mL/min, and detection wavelength of 270 nm. The gradient procedure was as follows: The mobile phase was composed of eluent A (0.1% formic acid in double distilled water) and eluent B (100% acetonitrile). Elution started at 10% of eluent B, raised to 35% in 4 min, and held for 1 min. Then, it decreased from 35% to 10% in 15 min and held for 10 min.

3 Results and discussion

3.1 Reducing HMF generation of corn stover in steam pretreatment

Unlike some past works [31, 32], little sucrose was found in this batch of corn stover, which was in agreement with some other previous researches [14, 20, 21]. Water-soluble glucose and fructose accounted for 5.7% of the dry solids in this batch of raw corn stover. However, certain amounts of sugars were degraded in the steam pretreatment operation and the HMF formation increased with the increasing temperature. Approximately 22.2% glucose and 22.6% fructose were degraded at 160 °C for 5 min, and 38.3% glucose and 44.8% fructose were degraded at 180 °C for 5 min (Fig. 1).

To reduce the water-soluble carbohydrate (WSC) degradation, lactic acid solid-state fermentation was performed by inoculation of lactic acid bacteria onto corn stover prior to steam pretreatment. Past work has shown that the addition of this extra step did not lead to the evident increase of the cost since it was conducted at the storage region for 7 days without extra reactor used [21]. Meanwhile, no nutrient addition was needed because of the existence of sufficient nitrates, trace elements, and vitamins in corn stover [1, 6, 7, 24, 33]. Only lactic acid bacteria seeds cover material for anaerobic condition, and additional operators were needed in industrial-scale applications [21].

A total of 25.7 mg/g DM of lactic acid and 6.6 mg/g DM of acetic acid were produced with the conversion of 38% glucose (24.8-15.5 mg/g DM) and 79% fructose (32.0-6.7 mg/g DM) after 7 days' anaerobic solid-state fermentation at storage period (Fig. 2). The reason for the incomplete conversion was that the solid-state fermentation was conducted at high solids loading without well-controlled environment such as insufficient mass and heat transfer, declined pH caused by the lactic acid generation, and poorly dispersed seed inoculation. Further increase of the conversion yield certainly led to the cost increase such as supplement of neutralizer (ammonia); therefore, natural condition without extensive modification was chosen [21].

The HMF generation in the steam-treated corn stover was significantly reduced in the range of 24.5%-47.5% under varying temperatures (140-180 °C). Other inhibitor compounds such as furfural and phenolic compounds were essentially the same as those in the regular steam pretreatment (Table 1). The results show that HMF formation in steam pretreatment was effectively reduced by converting the WSC into L-lactic acid before pretreatment.

Raw corn stover and lactic acid-containing corn stover were steam treated for 5 min at different reaction temperatures (120, 140, 160, 180 °C). The composition was defined as milligram components in the per gram dry corn stover. The experiments were carried out in duplicate and the data used here was taken from the average of two parallel experiments.

3.2 Elevating hydrolysis yield of steam-treated corn stover

Steam pretreatment could effectively open up the intact lignocellulosic matrix. The hemicellulose (xylan and arabinan) content decreased with the increasing temperature (120, 140, 160, 180 °C) (Fig. 1a), and the generation of oligosaccharides (xylooligomer and arabinooligomer) and monosaccharides (xylose and arabinose) increased correspondingly (Fig. 1b and c), while the cellulose content maintained relatively constant (Fig. 1a). Enzymatic hydrolysis yield of cellulose

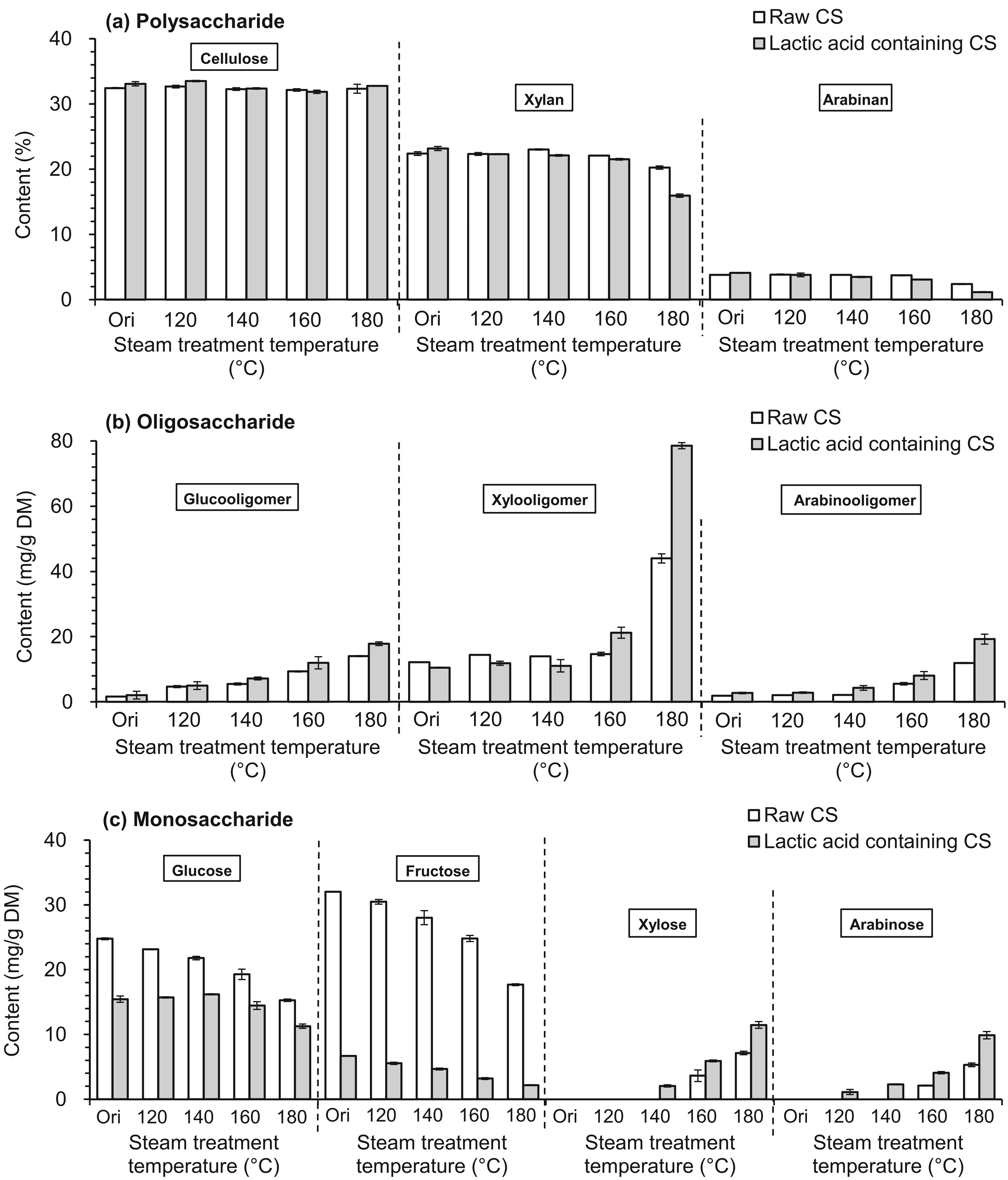

Fig. 1 Polysaccharide, oligosaccharide, and monosaccharide of steam-treated corn stover. **a** Polysaccharide. **b** Oligosaccharide. **c** Monosaccharide. Raw corn stover and lactic acid-containing corn stover were steam treated for 5 min at different reaction temperatures (120, 140, 160, 180 °C). Ori, raw corn stover, and lactic acid-containing corn stover without steam treated. The composition was defined as milligram components in the per gram dry corn stover. The experiments were carried out in duplicate and the data used here was taken from the average of two parallel experiments. CS, corn stover

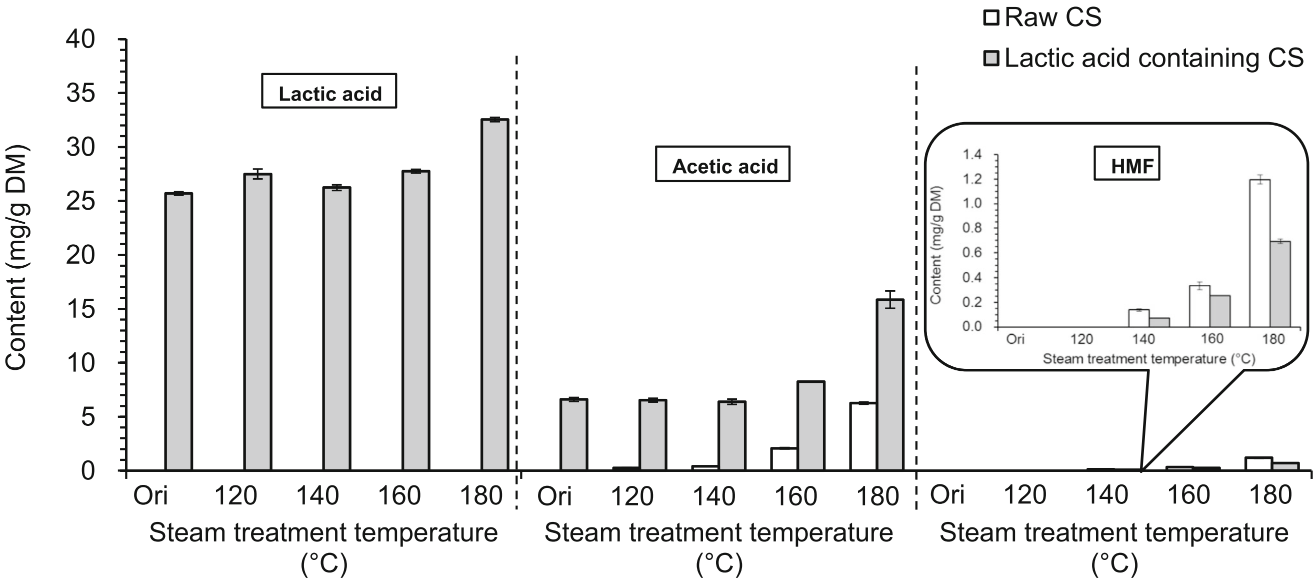

Fig. 2 Acids and HMF of steam-treated corn stover. Raw corn stover and lactic acid-containing corn stover were steam treated for 5 min at different reaction temperatures (120, 140, 160, 180 °C). Ori, raw corn stover, and lactic acid-containing corn stover without steam treated. The composition was defined as milligram components in the per gram dry corn stover. The experiments were carried out in duplicate and the data used here was taken from the average of two parallel experiments. CS, corn stover

increased with the increasing temperature (Fig. 3), indicating that steam treatment could effectively remove hemicellulose barrier and increase enzymatic hydrolysis of lignocellulose biomass.

The pH of corn stover decreased from 6.23 to 4.27 after bioconversion of WSC in lactic acid solid-state fermentation (Table 1). The generated lactic acid and byproduct acetic acid were expected to catalyze the hydrolysis of hemicellulose and acetyl groups in steam pretreatment. The upgraded steam pretreatment, i.e., lactic acid-assisted steam pretreatment, showed increased hemicellulose conversion yield and hemicellulose-derived sugars generation compared to regular steam treatment (28.8% vs. 9.6% of xylan conversion and 69.6% vs. 37.2% of arabinan conversion at 180 °C), suggesting that the 2.57% (w/w) endogenous-generated lactic acid could promote the hydrolysis of hemicellulose in steam pretreatment. Moreover, the enzymatic hydrolysis yield of cellulose also increased by 5.4%-16.6% compared to that after regular steam treatment at corresponding treated temperatures (140-180 °C) (Fig. 3).

Overall, the lactic acid-assisted steam pretreatment had better pretreatment efficiency than regular steam pretreatment, including increased hemicellulose conversion yield and hemicellulose-derived sugars generation, increased enzymatic hydrolysis yield of cellulose, and decreased HMF formation.

Table 1 Furfural, phenolic compounds, and pH of steam-treated corn stover

Pretreatment conditions	Furfural (mg/g DM)	4-Hydroxy-benzaldehyde (mg/g DM)	Vanillin (mg/g DM)	Syringal-dehyde (mg/g DM)	pH
(A) Steam pretreatment					
Raw corn stover	0.00 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.12 ± 0.01	6.23 ± 0.03
120 °C	0.00 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.13 ± 0.01	5.87 ± 0.01
140 °C	0.00 ± 0.00	0.04 ± 0.01	0.06 ± 0.02	0.15 ± 0.00	5.71 ± 0.01
160 °C	0.00 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.17 ± 0.01	5.76 ± 0.00
180 °C	0.37 ± 0.01	0.31 ± 0.04	0.28 ± 0.02	0.41 ± 0.04	5.05 ± 0.00
(B) Lactic acid-assisted steam pretreatment					
Lactic acid-containing corn stover	0.00 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.09 ± 0.01	4.27 ± 0.01
120 °C	0.00 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.09 ± 0.00	4.30 ± 0.01
140 °C	0.00 ± 0.00	0.07 ± 0.01	0.05 ± 0.01	0.12 ± 0.01	4.30 ± 0.00
160 °C	0.08 ± 0.00	0.10 ± 0.01	0.11 ± 0.01	0.19 ± 0.02	4.32 ± 0.01
180 °C	0.41 ± 0.02	0.35 ± 0.03	0.34 ± 0.03	0.44 ± 0.02	4.19 ± 0.01

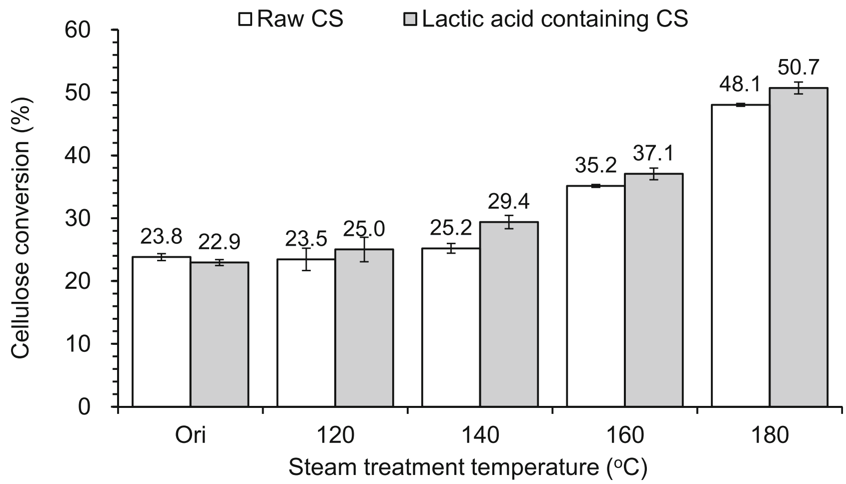

Fig. 3 Cellulose hydrolysis yield of steam-treated corn stover. Conditions: the enzymatic hydrolysis was conducted at 2.5% (w/w) solids loading, 20 FPU/g DM (26 mg cellulase protein/g cellulose), pH 6.0, and 39 °C for 48 h. The experiments were carried out in duplicate and the data used here was taken from the average of two parallel experiments. CS, corn stover

The lactic acid-assisted steam pretreatment was expected to be applied in both biorefinery plants and ruminant feed plants.

In cellulosic lactic acid biorefinery process, our past work had shown that the increased pretreatment efficiency and produced lactic acid could enhance the biorefinery efficiency and lactic acid yield [21], while in ruminant feed production process, the increased hemicellulose conversion yields, hemicellulose-derived sugars generation, and the cellulose enzymatic hydrolysis yield could be helpful to increase the digestibility of corn stover as ruminant feed and provide more volatile fatty acids and protein for ruminants [9, 34, 35]. Also, lactic acid-assisted steam pretreatment generated less toxic HMF and might weaken its negative effect for rumen microorganisms, ruminants, and workers [12, 19, 36]. Moreover, the generated lactic acid and acetic acid maintained stable in steam pretreatment, and could consequently serve as energy provision for ruminants [11, 37, 38], which increased the total energy utilization of corn stover. Although the actual conversion of corn stover in cattle rumens is more complicated, the present results have provided a strong evidence on increasing cellulose and hemicellulose digestion in the simulation of rumen environment. Overall, this study upgrades regular steam pretreatment to lactic acid-assisted steam pretreatment that facilitates potential applications in biorefinery and ruminant feed production.

4 Conclusion

In this study, corn stover was fermented at solid state to convert water-soluble carbohydrates into lactic acid prior to steam pretreatment. Compared to the regular steam pretreatment at the same severity (180 °C for 5 min), the conversion yields of xylan and arabinan in the steam pretreatment were both increased from 9.6% and 37.2% to 28.8% and 69.6%, respectively. Cellulose conversion also increased from 48.1% to 50.7% in the subsequent enzymatic hydrolysis. On the other hand, the generation of the toxic HMF reduced by 42.1% due to the pre-conversion of WSC into the relatively thermal stable lactic acid. Lactic acid-assisted steam pretreatment showed great potentials in processing corn stover with higher hydrolysis yield and lower inhibitors generation.

Funding This study was funded by the National Natural Science Foundation of China (31961133006, 21978083) and the National Key Research and Development Program of China (2017YFB0309302).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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