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Re-examination of dilute acid hydrolysis of lignocellulose for production of cellulosic ethanol after de-bottlenecking the inhibitor barrier

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ABSTRACT

Dilute acid hydrolysis of lignocellulose biomass had been used for production of cellulosic ethanol since 1940 s. The major technical barrier is the acid catalyzed dehydration of monosaccharides to furan aldehydes (furfural and 5-hydroxymethylfurfural), resulting in the high loss of fermentable sugars and significant inhibition on the fermentability of ethanologenic strains. This study re-examined the dilute acid hydrolysis of corn stover and cellulosic ethanol fermentation after a novel biodetoxification approach was introduced to de-bottleneck the inhibitor barrier. The cocktail of sulfuric acid, phosphoric acid and oxalic acid hydrolyzed corn stover to the 51.1 g/L of glucose (0.50 g/g cellulose) and 18.1 g/L of xylose (0.22 g/g xylan). The furfural, 5-hydroxymethylfurfural and acetic acid in the corn stover hydrolysate were completely removed by *Paecilomyces variotii* FN89, leading to the successful ethanol fermentation of 24.2 g/L, corresponding to 72.6 kg per metric ton of dry corn stover. No wastewater streams, solid wastes and toxic compounds were generated in hydrolysis, biodetoxification and fermentation. The techno-economic evaluations suggest that the cost reduction of replacing cellulase enzyme with cheap acid catalysts compensated the partial ethanol loss of sugar conversion to inhibitors (21.5–89.1%). The re-examination of acid hydrolysis process reveals that a substantial breakthrough in highly active and selective acid catalyst is required for acid hydrolysis to compete with enzymic hydrolysis for cellulosic ethanol fermentation.

1. Introduction

Cost-effective hydrolysis of cellulose and hemicellulose carbohydrates into fermentable sugars is crucially important for commercialization of cellulosic ethanol and biobased chemicals (Hassan et al., 2018). The most commonly applied hydrolysis methods include acid hydrolysis and enzymatic hydrolysis (Huang and Fu, 2013; Taherzadeh and Karimi, 2007). Enzymatic hydrolysis currently is the dominant choice of biorefinery process by its high selectivity for fermentable sugars at mild reaction conditions (Shuai and Pan, 2012). However, the high cellulase enzyme cost presents the high percentage of the overall cost of cellulosic ethanol despite the great significant efforts on cost reduction by microbial modification, enzyme recycling, novel enzyme and accessory discovery (Brijwani et al., 2010; Champreda et al., 2019; Gregg et al., 1996; Kim et al., 2015; Xu et al., 2019). The purchase mode leads to an enzyme cost at least $2.71/gal ethanol, accounting for 50% of cellulosic ethanol cost, while the on-site enzyme mode is lack of commercial verification for its cost-effectiveness (Klein-Marcuschamer et al., 2012; Liu et al., 2016). An extra acid hydrolysis step (pretreatment) is also required to overcome the bio-recalcitrance of lignocellulose before enzymatic hydrolysis (Gong et al., 2021), while the inhibitory compounds generated from pretreatment require an extra detoxification step to be removed (Jing et al., 2009; Palmqvist and Hahn-Hägerdal, 2000). Totally, the front to end processes including pretreatment, fractionation and enzymatic hydrolysis account for at least 60% of the total cellulosic ethanol cost (Gregg et al., 1998).

Acid hydrolysis by mineral or organic acid catalysts is the traditional method for production of cellulosic ethanol since 1940 s (Murphy, 1945), but was almost abandoned in the past few decades mainly due to the formation of undesirable furan aldehydes from monosaccharides (furfural from xylose and 5-hydroxymethylfurfural from glucose) and weak organic acids by-products (Huang and Fu, 2013; Taherzadeh and Karimi, 2007). These undesired reactions not only reduce the fermentable sugars yields, but also generate the inhibitory compounds to ethanologenic microbes (Shuai and Pan, 2012). These high concentrated inhibitors are unable to be effectively removed by conventional

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detoxification methods such as water washing, activated carbon adsorption, reaction conversion, extraction etc. (Jönsson et al., 2013). Ultimately, the acid hydrolysate is almost worthless for any consequent bioconversion due to the highly toxicity to all most ethanologenic microbes.

A new breakthrough in biorefinery technology may provide a big opportunity for survival of dilute acid hydrolysis for cellulosic ethanol production. The newly isolated biodetoxification filamentous fungi, *Amorphotheca resinae* ZN1 and *Pacilomyces variotii* FN89, are capable of completely and ultimately degrading the lignocellulose-derived inhibitors to CO_2 and H_2O without consumption of fermentable sugars (Dong and Bao, 2010; Yi et al., 2019; Zhang et al., 2021a, 2010). This biodetoxification approach facilitates the biorefinery processing into a similar fashion with corn dry milling in almost measurable aspects (Liu and Bao, 2017; Zhang et al., 2021a). We expect that this biodetoxification fungi should also be capable of degrading the highly concentrated inhibitors in dilute acid hydrolysate, and the sugars in biodetoxified hydrolysate can be converted into ethanol in the consequent fermentation step. The further advances in acid hydrolysis catalyzed by combinational cocktails of mineral and organic acids could also deliver the higher reactivity and lower inhibitor generations than that of the mineral acid catalysts only (Guo et al., 2012; Pal et al., 2016b; Schreiner, 2010).

In this study, the dilute acid hydrolysis of corn stover were optimized by adjusting the acid cocktails as well as temperature, solids loading, and reaction time. The obtained hydrolysate was biodetoxified using strain *Paecilomyces variotii* FN89 to remove the highly concentrated inhibitors, then used for cellulosic ethanol fermentation. Finally, the feasibility of current dilute acid hydrolysis after breaking the inhibitor barrier by biodetoxifications was evaluated compared with enzymatic hydrolysis process in three different scenarios.

2. Materials and methods

2.1. Raw feedstock

The raw corn stover was harvested in spring 2018, Nanyang city, Henan province, China. The raw feedstock was water washed, air dried, and milled to pass through the mesh with 10 mm in diameter. The raw feedstock was determined to contain 11.8% (w/w) of H_2O. The main compositions of the dry corn stover were 28.4 ± 2.3% of cellulose, 22.6 ± 0.7% of xylan, 18.2 ± 1.5% of lignin, and 7.4 ± 1.1% of ash. The other compositions include some soluble monosaccharides, protein, hemicellulose fractions (arabinan, mannan, galactan, acetyl groups), and unknown soluble solids (Aden et al., 2002; Han et al., 2018).

2.2. Reagents

Yeast extract (LP0021B) was purchased from Oxoid Co., Ltd, UK. Oxalic acid dihydrate was purchased form Titan scientific Co., Ltd, Shanghai, China. Sulfuric acid, phosphoric acid, and other chemical reagents were purchased from Sinopharm Chemical reagent Co., Ltd, China.

2.3. Microorganisms and medium

The biodetoxification strain *Paecilomyces variotii* FN89 (CGMCC 17665) was used for the removal of acetate, HMF and furfural in acid hydrolysates (Zhang et al., 2021a). *P. variotii* FN89 was preserved on potato dextrose agar (PDA) and cultured in the general synthetic medium (SM) containing 20 g/L glucose, 1 g/L yeast extract, 2 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, and 1 g/L $MgSO_4.7\ H_2O$.

The ethanol producing strain *Saccharomyces cerevisiae* XH7 was activated in YPD medium containing 2 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 2 g/L $MgSO_4.7\ H_2O$, and 10 g/L yeast extract (Li et al., 2016). The nutrients for ethanol fermentation consist of 2 g/L $(NH_4)_2SO_4$, 2 g/L $MgSO_4.7\ H_2O$, and 10 g/L yeast extract.

2.4. Dilute acid hydrolysis

Dilute acid hydrolysis of corn stover was conducted in a 20-L reactor equipped a helical impeller according to the previous protocols (Liu and Bao, 2017). Corn stover and acid solution were simultaneously fed into the reactor at the solid-liquid ratio of 1.5:1–1:10, and mixed for 5 min. The steam was then injected, and the hydrolysis was carried out at 175–195 °C for 5–25 min. The obtained hydrolysate was discharged from the bottom outlet of the reactor.

2.5. Semi-continuous submerged biodetoxification

The biodetoxification strain *P. variotii* FN89 was firstly cultured on PDA plate at 37 °C for 4 days. The spores were then washed and collected by 0.05% (w/w) tween 80 solution. The concentration of spore suspension was 10^9 spores/mL. The biodetoxification seed was prepared by inoculating 1% (v/v) of spore suspension into 100 mL of general SM medium in 500 mL shake flask, and culturing at 37 °C, 200 rpm for 18 h.

The pH value of hydrolysate was neutralized to 4.8 by calcium carbonate. The fresh acid hydrolysate contained high concentrated inhibitors (~10 g/L of acetate, 3 g/L of HMF, and 4 g/L of furfural). The hydrolysate was diluted by 40% (v/v) and then detoxified by semi-continuous fermentation. For the first batch submerged biodetoxification, 600 mL of hydrolysate was diluted with 400 mL of the SM medium containing 40 g/L of glucose and 20 g/L of xylose in a 3-L bioreactor. The biodetoxification seed was then inoculated at 20% (v/v) inoculum. The mixture was incubated at 37 °C, 300 rpm, 1 vvm for 24 h without adding nutrients till no acetate, HMF and furfural were detected. For the subsequent semi-continuous biodetoxification, 40% (v/v) of the biodetoxified hydrolysate was removed and the same amount of the fresh hydrolysate was fed every 24 h. The biodetoxified hydrolysate was collected and then maintained at 50 °C, 100 rpm under anaerobic conditions for 12 h in order to inactivate the biodetoxification strain *P. variotii* FN89 (Zhang et al., 2021b).

2.6. Ethanol fermentation

The ethanol-producing strain *S. cerevisiae* XH7 was activated in YPD medium at 30 °C, 180 rpm for 12 h. 10% (v/v) of YPD culture was inoculated into inactivated biodetoxified hydrolysate, and then incubated at 30 °C, 180 rpm for 24 h as ethanol fermentation seed. The ethanol fermentation began with inoculating 10% (v/v) of *S. cerevisiae* XH7 seed and adding nutrients. The anaerobic ethanol fermentation was carried out at 200 rpm, 30 °C for 72 h.

2.7. Yield and conversion rate calculations

The yield of glucose (g/g cellulose) or xylose (g/g xylan) hydrolyzed form cellulose or xylan can be calculated by the following Eq. (1):

$$\text{Sugar yield}(g/g) = \frac{C_1 \times m_1 \times (1 - D)}{m_0 \times C_0} \quad (1)$$

where C_1 is the sugar concentration in acid hydrolysate, m_1 is the weight of one batch hydrolysate, D is the solids content of hydrolysate. We approximate the density of the liquid fraction in hydrolysate as 1 g/mL. m_0 is the weight of raw corn stover on dry base, C_0 is the content of cellulose or xylan of raw corn stover on dry base.

The conversion ratio (%) of cellulose can be calculated by Eq. (2):

$$\text{Conversion ratio}(\%) = \frac{m_0 \times C_0 - m_1 \times D \times [C_1]}{m_0 \times C_0} \quad (2)$$

where $[C_1]$ is the cellulose content of solids residue in hydrolysate.

2.8. Analysis method

The compositions of feedstock were determined by a two-step acid hydrolysis method described by NREL reports (Sluiter et al., 2008, 2012). The concentrations of glucose, xylose, acetic acid, and ethanol were measured by HPLC method (Liu and Bao, 2017). Furan and phenolic compounds were analyzed according to the method of Yi et al. (2019).

2.9. Materials mass balance and techno-economic evaluation

The techno-economic evaluations are based on the construction and operation of the biorefinery plant processing 300,000 tons of dry corn stover per year, equivalent to the average capability to the petroleum refining plant (Liu and Bao, 2019). The exchange rate from US dollar ($) to Chinese Yuan (CNY) is set to 1:6.38 (http://data.stats. gov.cn/).

3. Results and discussion

3.1. Examination of combined acid catalysts for corn stover hydrolysis

Acid catalyst effectively breaks the glycosidic bonds of cellulose, but not selective for polysaccharide (cellulose and hemicellulose) or monosaccharides (glucose and xylose). This poor selectivity results in the over-degradation of glucose and xylose into furan aldehydes (HMF and furfural) and organic acids (formic acid and levulinic acid)

Table 1
Maximizing the fermentable sugars yield by varying hydrolysis parameters.

	Glucose (g/L)	Cellulose conversion (%)	Glucose yield (g/g cellulose)	Xylose (g/L)	Xylose yield (g/g xylan)	Acetate (g/L)	HMF (g/L)	Furfural (g/L)
(a) Temperature (°C)	(20 min; 1200 g dry CS (60% loading, w/w); 8.5% sulfuric acid based on dry CS)							
175	8.2 ± 2.5	35.5	0.04	35.4 ± 1.3	0.21	8.1 ± 0.0	3.6 ± 1.2	3.3 ± 0.1
185	24.1 ± 1.2	38.8	0.15	10.3 ± 0.0	0.08	9.1 ± 0.0	4.5 ± 0.0	4.1 ± 0.0
195	20.4 ± 2.0	55.3	0.15	4.8 ± 0.2	0.04	10.6 ± 0.1	5.00 ± 0.5	2.5 ± 0.0
(b) Acids cocktail (% DM)	(185 °C; 20 min; 1200 g dry CS (60% loading, w/w); SA, sulfuric acid; OA, oxalic acid; PA, phosphoric acid)							
8.5%SA	18.6 ± 2.8	51.6	0.13	7.0 ± 0.2	0.06	9.1 ± 0.3	4.4 ± 0.2	3.8 ± 0.2
8.5%OA	17.4 ± 0.7	50.5	0.13	12.3 ± 1.4	0.12	9.5 ± 0.2	2.1 ± 0.1	3.0 ± 0.5
8.0%SA+ 0.5% PA	19.6 ± 1.0	46.0	0.15	9.3 ± 0.4	0.09	9.4 ± 0.4	4.1 ± 0.1	4.0 ± 0.1
6.0%SA+ 0.5%PA+ 2.0% OA	39.6 ± 0.5	54.0	0.29	9.5 ± 0.1	0.09	10.7 ± 0.4	4.9 ± 0.1	3.4 ± 0.2
6.0%SA+ 0.5%PA+ 6.0% OA	40.8 ± 1.4	64.2	0.31	10.3 ± 0.3	0.10	10.9 ± 0.4	4.8 ± 0.2	2.8 ± 0.1
8.0%SA+ 0.5%PA+ 2.0% OA	34.4 ± 2.2	65.2	0.26	8.8 ± 0.7	0.08	10.9 ± 0.8	4.1 ± 0.4	2.6 ± 0.1
8.0%SA+ 0.5%PA+ 6.0% OA	30.6 ± 1.2	78.0	0.22	7.7 ± 0.6	0.07	10.4 ± 1.0	3.9 ± 0.1	2.9 ± 0.0
(c) Solids loading (%, w/w)	(185 °C; 20 min; 167, 333, 500, 667, 833 g dry CS; 6.0% SA + 0.5% PA + 6.0% OA.*ND, not detected)							
10	3.0 ± 0.2	56.7	0.12	7.8 ± 0.4	0.39	1.6 ± 0.6	0.2 ± 0.0	ND*
20	10.0 ± 0.4	67.1	0.21	12.6 ± 0.5	0.34	3.8 ± 0.5	0.8 ± 0.0	0.8 ± 0.0
30	18.8 ± 0.5	67.8	0.27	15.1 ± 0.4	0.27	5.9 ± 0.1	1.5 ± 0.1	1.8 ± 0.2
40	33.8 ± 1.9	65.2	0.44	12.7 ± 1.5	0.20	8.5 ± 0.0	3.0 ± 0.1	2.5 ± 0.0
50	36.6 ± 1.2	72.3	0.32	15.3 ± 0.2	0.17	8.6 ± 0.3	3.6 ± 0.1	2.4 ± 0.1
(d) Catalysts dosage (% DM)	(185 °C; 20 min; 667 g dry CS (40% solids, w/w))							
4.25%SA+ 0.5% PA+ 4.25%OA	26.1 ± 0.7	52.6	0.33	23.6 ± 0.7	0.38	7.9 ± 0.6	2.1 ± 0.0	2.6 ± 0.0
7.25%SA+ 0.5% PA+ 7.25%OA	39.4 ± 0.8	73.0	0.49	13.4 ± 0.4	0.21	9.1 ± 0.2	3.5 ± 0.1	2.7 ± 0.3
8.75%SA+ 0.5% PA+ 8.75%OA	34.9 ± 0.7	71.6	0.48	11.1 ± 0.5	0.19	9.3 ± 0.2	4.4 ± 0.1	2.7 ± 0.2
(e) Reaction time (h)	(185 °C; 20 min; 667 g dry CS (40% solids, w/w); 7.25% SA + 0.5% PA + 7.25%OA)							
10	49.1 ± 0.5	44.0	0.40	23.2 ± 0.1	0.24	10.1 ± 0.7	2.9 ± 0.0	3.7 ± 0.1
15	39.7 ± 2.3	47.1	0.43	18.5 ± 1.0	0.25	8.8 ± 0.0	2.8 ± 0.2	2.9 ± 0.0
25	26.4 ± 0.3	74.4	0.41	10.0 ± 0.1	0.20	7.3 ± 0.2	3.1 ± 0.0	1.9 ± 0.0
5 + 15*	39.3 ± 1.5	63.4	0.35	18.5 ± 0.9	0.20	9.2 ± 1.3	2.5 ± 0.1	3.6 ± 0.3
10 + 10*	46.8 ± 1.7	66.3	0.42	19.8 ± 0.6	0.23	11.1 ± 1.0	3.9 ± 0.7	4.4 ± 0.0
15 + 5*	51.1 ± 1.7	72.7	0.50	18.1 ± 0.7	0.22	10.9 ± 0.2	4.4 ± 0.2	4.3 ± 0.2

* Reaction time 5 + 15, 10 + 10, 15 + 5 indicate that the hydrolysis was maintained for 5, 10, 15 min at 185 °C, then the heating steam supply was stopped and kept being for another 15, 10, 5 min, in which the temperature was still 174.3, 178.8, and 182.1 °C before the hydrolysis ended, respectively.

(Taherzadeh et al., 2000). The first step of this study was to examine the proper catalyst combination and hydrolysis parameters to obtain the better yield of fermentable sugars (Table 1).

Higher temperature accelerates both the hydrolysis rate of cellulose/hemicellulose into glucose/xylose and the degradation rate of glucose/xylose into HMF/furfural (Taherzadeh and Karimi, 2007; Taherzadeh et al., 1999). Table 1a shows that the relatively more glucose was obtained at 185 °C from corn stover at the moderate sulfuric acid dosage (8.5% of dry corn stover weight) and high solids loading (60%, w/w).

The mixed acid cocktails of organic and inorganic acids have been applied by its merits on reactivity and selectivity (Guo et al., 2012; Pal et al., 2016a, 2016b). Table 1b designed a combination of sulfuric acid, oxalic acid, and phosphoric acid for dilute acid hydrolysis of corn stover. Sulfuric acid is a commonly used mineral acid for lignocellulose hydrolysis (Pal et al., 2016b). Oxalic acid is a biodegradable dicarboxylic acid with strong acidity on β-1, 4-glucosidic bonds while generates less furan aldehyde during the hydrolysis (Qing et al., 2015; Zhang et al., 2013). Phosphoric acid hydrolyzes the hemicellulose with low furfural generation and the phosphates after hydrolysis reaction is used as ethanol fermentation nutrients (de Vasconcelos et al., 2013; Martínez-Patiño et al., 2015). The dosage of phosphoric acid was set to 0.5% of dry corn stover weight to match the phosphate addition (2 g/L KH_2PO_4) in the subsequent ethanol fermentation. The similar glucose production (18.6 ± 2.8 vs. 17.4 ± 0.7 g/L) and yield (0.13 vs. 0.13 g/g cellulose) were obtained at the same dosage of sulfuric acid or oxalic acid (8.5% of dry corn stover weight), while more xylose was preserved with oxalic acid than sulfuric acid (0.12 vs. 0.06 g/g xylan) and less HMF and furfural were generated (53.3% and 19.3% reduction, respectively). The cocktail of sulfuric acid (8.0%) and phosphoric acid (0.5%) hydrolyzed corn stover with the same efficiency of sulfuric acid (8.5%). The cocktail of the three acids (6.0% sulfuric acid, 0.5% phosphoric acid, and 6.0% oxalic acid) gave the improved glucose yield of 0.31 g/g cellulose.

The acid dosage was re-optimized after the optimal initial solids loading decreased from 60% to 40% (w/w) (Tables 1c and 1d). Because the acid dosage was based on dry corn stover matter, thus the lower initial solids loading led to the reduction in the amount of acid used in the overall hydrolysis. The initial solids loading and acid dosage (40% (w/w) solids loading with 7.25% sulfuric acid, 0.5% phosphoric acid, and 7.25% oxalic acid of dry corn stover weight) led to the highest glucose yield of 0.49 g/g cellulose (Tables 1c and 1d). By the reaction time control (15 min then stop the steam input), the glucose and xylose production increased to 51.1 ± 1.7 g/L and 18.1 ± 0.7 g/L, with the generation of acetic acid, HMF, and furfural of 10.9 ± 0.2 g/L, 4.4 ± 0.2 g/L, and 4.3 ± 0.2 g/L, respectively. The conversion ratio of cellulose was 72.7% and xylan was almost 100%, but the yields were only 0.50 g glucose/g cellulose and 0.22 g xylose/g xylan (Table 1e). Generally, the enzymatic hydrolysis at high solids loading is incomplete due to the irreversible and non-productive adsorption of cellulase onto lignin (Chandra et al., 2011). This study shows that the incomplete hydrolysis of cellulose (25.6–64.5%) also occurred in dilute acid hydrolysis at high solids loading.

3.2. Biodetoxification and subsequent ethanol fermentation

The previous studies revealed that the dilute acid hydrolysates of corn stover were essentially not capable of direct use for ethanol fermentation due to lack of available means to remove the high concentrated inhibitors. Only little ethanol (less than 5 g/L) was generated from undetoxified acid hydrolysate (Brandberg et al., 2005; Klinke et al., 2004). The complete removal of the highly concentrated inhibitors in the dilute acid hydrolysate is a prerequisite step for achieving high ethanol fermentation performance. This study used the newly isolated biodetoxification fungi for selectively and completely degrading all spectrum of lignocellulose-derived inhibitors while the fermentable sugars were still well preserved (Zhang et al., 2021a).

The corn stover hydrolysate by mixed acids hydrolysis was neutralized to pH 4.8 by adding $CaCO_3$, then the semi-continuous fermentation was applied by inoculating *P. variotii* FN89 fungus for the biodetoxification. The neutralization partially reduces furan and phenolic aldehydes into their corresponding alcohols (Table 2). The further investigation showed that *P. variotii* FN89 was unable to survive in the hydrolysate unless the hydrolysate was diluted by 40%, at which *P. variotii* FN89 degraded all the acetate, HMF, and furfural with the minimum fermentable sugar loss of 11.5 ± 0.7% (Table 3).

The semi-continuous biodetoxification not only reduced the initial inhibitors concentration by discharging/feeding operations, but also adaptively evolved the tolerance of the strain to the inhibitors. Fig. 1a shows the semi-continuous submerged biodetoxification of corn stover hydrolysate by *P. variotii* FN89. The initial acetic acid, HMF and furfural in every batch were 6.3 ± 0.3 g/L, 2.3 ± 0.1 g/L and 2.3 ± 0.2 g/L, respectively, and the final titers were reduced to zero (not detectable on HPLC) within 24 h. The final glucose and xylose were 50.3 ± 4.5 g/L and 18.4 ± 2.0 g/L, respectively, with less than 11.6% loss of the total fermentable sugars. After the biodetoxification, the corn stover hydrolysate was maintained at 50 °C for 12 h for heat inactivating the biodetoxification fungus (Zhang et al., 2021b). Fig. 1b shows the inoculation of *S. cerevisiae* XH7 and the ethanol titer reached to 24.2 ± 0.3 g/L after 60 h with the complete consumption of glucose and 6.9 ± 0.3 g/L of xylose residual. The overall yield of ethanol reached 94.8% of the theoretical yield based on the sugar conversion in the biodetoxified corn stover hydrolysate. The residual aromatic aldehydes such as vanillin, syringaldehyde, hydroxybenzaldehyde from the lignin could be responsible to the incomplete xylose consumption (Table 2) (Klinke et al., 2004; Yi et al., 2019).

3.3. Mass balance and techno-economic evaluation

The overall mass balance of the current dilute acid hydrolysis process from 1000 kg dry corn stover to ethanol was calculated based on the experimental results (Fig. 2). 72.6 kg of ethanol was produced by dilute acid hydrolysis, biodetoxification and ethanol fermentation from 283.8 kg of cellulose and 225.9 kg of xylan in 1000.0 kg of dry corn stover. The ethanol yield is only about 35% of dry biorefinery process with enzymatic hydrolysis (Liu and Bao, 2017). No wastewater streams,

Table 2
The concentrations of main phenolic aldehyde inhibitors in original hydrolysate, neutralized hydrolysate, and biodetoxified hydrolysate.

	Original hydrolysate	Neutralized hydrolysate	Biodetoxified hydrolysate[a]
HMF	4.43 ± 0.23	3.74 ± 0.01	ND
HMF alcohol	ND[b]	0.81 ± 0.01	ND
HMF acid	ND	ND	ND
Furfural	4.34 ± 0.34	3.67 ± 0.14	ND
Furfuryl alcohol	ND	0.69 ± 0.32	ND
Furoic acid (g/L)	ND	ND	0.72 ± 0.33
4-HBA (g/L)[c]	0.56 ± 0.06	0.45 ± 0.02	0.07 ± 0.04
4-HBA alcohol (g/L)	ND	0.08 ± 0.01	0.24 ± 0.12
4-HBA acid (g/L)	ND	ND	0.35 ± 0.16
Vanillin (g/L)	1.17 ± 0.06	0.66 ± 0.03	0.12 ± 0.04
Vanillyl alcohol (g/L)	ND	0.40 ± 0.02	0.79 ± 0.31
Vanillic acid (g/L)	ND	ND	0.78 ± 0.58
Syringaldehyde (g/L)	2.31 ± 0.12	1.91 ± 0.09	0.27 ± 0.04
Syringyl alcohol (g/L)	ND	0.55 ± 0.04	0.49 ± 0.20
Syringic acid (g/L)	ND	ND	0.05 ± 0.00

[a] The biodetoxified hydrolysate was obtained from the semi-continuous biodetoxification process as shown in Fig. 2.
[b] ND, not detected by HPLC.
[c] 4-HBA, 4-hydroxybenzaldehyde.

Table 3
The biodetoxification performance of the corn stover hydrolysate by mixed acids hydrolysis with different dilution rate by *P. variotii* FN89.

Dilution[a] (%)	Acetate (g/L)	HMF (g/L)	Furfural (g/L)	Biodetoxification time[b] (h)	Sugar loss[c] (%)
No dilution	10.9 ± 0.2	4.4 ± 0.2	4.3 ± 0.2	/	/
20%	8.8 ± 0.5	3.6 ± 0.1	3.4 ± 0.2	84	35.2 ± 2.3
40%	6.3 ± 0.3	2.3 ± 0.2	2.3 ± 0.1	24	11.5 ± 0.7
60%	4.2 ± 0.1	1.6 ± 0.3	1.7 ± 0.1	24	15.3 ± 0.9
80%	1.1 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	12	18.1 ± 1.2

[a] The hydrolysate was diluted with SM medium containing 40 g/L glucose and 20 g/L xylose.
[b] The biodetoxification was conducted in 500 mL flasks until no acetate, HMF and furfural was detected in the hydrolysate. The total liquid loading is 100 mL. Conditions: 37 °C, 200 rpm.
[c] The sugar loss includes the total loss of glucose and xylose.

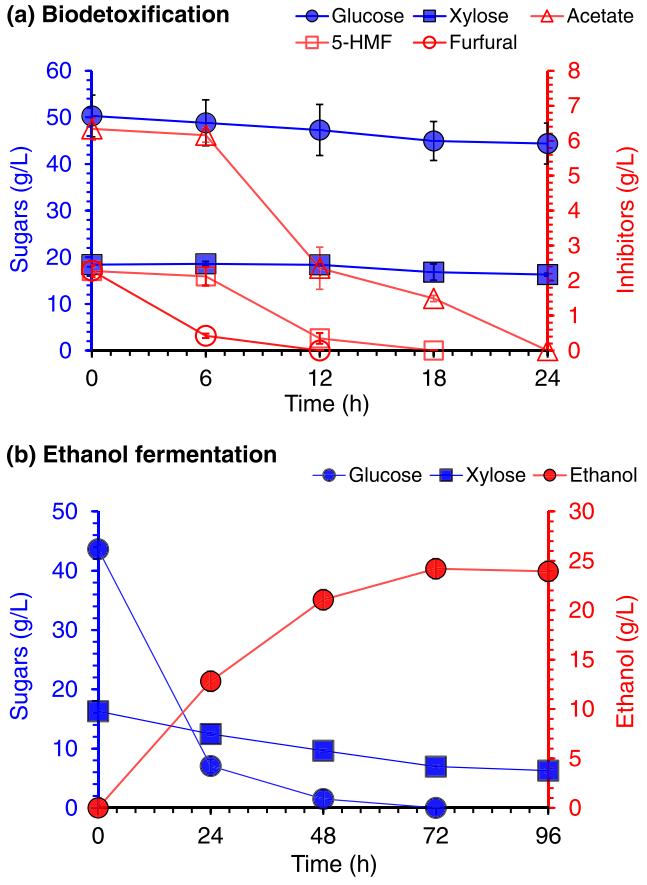

Fig. 1. Biodetoxification and consequent ethanol fermentation of corn stover hydrolysate by mixed acids hydrolysis. The acid hydrolysis was conducted at 185 °C for 15 min (then stop the steam input and kept for another 5 min) with 40% (w/w) initial solids loading, 7.25% sulfuric acid, 0.5% phosphoric acid, and 7.25% oxalic acid of dry corn stover weight as acids catalyst. The biodetoxification and ethanol fermentation were conducted in one single 3-L bioreactors. Only one batch fermentation process was shown. (a) Submerged liquid biodetoxification. 37 °C, 1 vvm aeration, 300 rpm stirring, 20% (v/v) inoculation, 2.5% dilution ratio (60% of broth discharging and fresh corn stover hydrolysate feeding every 24 h); (b) ethanol fermentation. 30 °C, 200 rpm, 10% (v/v) inoculation. The process of heat-inactivation at 50 °C for 12 h was not shown.

solid wastes and toxic compounds were generated before distillation separation step during the overall processing starting from raw corn stover. 1482.4 kg of solids residue and 2299.4 kg of wastewater were generated after distillation and S/L separation, equivalent to 20.4 and 31.7 g/g ethanol produced. The distilled water can be recycled to hydrolysis or fermentation processes.

The results show that the acid hydrolysis replaced the use of expensive cellulase enzyme with the cheap mineral and organic acids, but the ethanol yield was also significantly reduced comparing with that of enzymatic hydrolysis (Liu and Bao, 2017). A preliminary techno-economic evaluation was further conducted on the performances of dilute acid hydrolysis and enzymatic hydrolysis. The acid hydrolysis was represented by the results in Fig. 2 (combinational acid hydrolysis and complete removal of the inhibitors) (Case 1), and the enzymatic hydrolysis process was represented by the NREL process (Case 2) (Humbird et al., 2011), and the dry biorefining process (dry acid pretreatment, biodetoxification, simultaneous enzymatic saccharification and co-fermentation) (Case 3–4) (Liu and Bao, 2017; Liu et al., 2018), where Case 4 indicated the results with the reduced cellulase dosage from 10 to 5 mg/g cellulose (Table 4).

The overall materials balance and the preliminary techno-economic evaluation were established with the full-scale processing capacity of 300,000 tons of dry corn stover, equivalent to the average capability of the petroleum refining plant (Table 5) (Liu and Bao, 2019). For dilute acid hydrolysis processing (Case 1), the annual ethanol production is 7.19 million gallons, which is only 29.6–33.9% of the ethanol production by the general enzymatic hydrolysis processing (Case 2–3). The reduced ethanol production represents an annual economic loss of $31.0–37.7 million based on current ethanol sale price of $2.21/gal. The acid hydrolysis process saves $8.11 million per year of cellulase enzyme cost based on the cellulase price of $4.24/kg protein by on-site production of enzymes (Case 2) (Humbird et al., 2011), only covers 21.5% of the ethanol production loss even under the best performance of acid hydrolysis process. It is worth noting that this calculation is based on the on-site enzyme production which is lack of commercial verification. If the cellulase is supplied by the purchase from different commercial industrial enzyme makers (Case 3), the prices of cellulase will be increased to $6.27 or 23.3/kg protein and the annual cost of cellulase enzyme will reach to $7.43 or 27.62 million accordingly (Liu and Bao, 2017). There are significant fluctuations in the cellulase enzyme quotations provided by different cellulase suppliers. The wide price fluctuations and unaffordable cost by purchase mode of cellulase enzymes would raise a high uncertainty on sustainable and economical production of cellulosic ethanol. The further reduction the cellulase dosage from 10 to 5 mg protein/g cellulose (Case 3–4) results in 18.5% reduction of ethanol annul output with the economic loss of $8.67 million. And the annual cost of purchasing cellulase is corresponding reduced by $4.19 or 15.19 million. The current used low dosage of cellulase in high solids loading SSCF is generally 10 mg protein/g cellulose without adding any accessory enzymes and additives. On this basis, whether the dosage of cellulase can be reduce by sacrificing part of ethanol output is needed to be further weighed against the price of commercially available cellulase and economic loss of ethanol. Another uncertainty in dilute acid hydrolysis is the high acid catalyst dosage, whose annual cost is over 10.0 folds greater than that of dilute acid pretreatment in enzymatic hydrolysis processing (Table 5).

In summary, the dilute acid hydrolysis process for cellulosic ethanol production was successfully achieved by mixed acids hydrolysis and unique biodetoxification method. The re-examination of the current acid hydrolysis process, the best results of acid hydrolysis process up to date, reveals that the acid hydrolysis process is still not competitive with that of enzymatic hydrolysis process after the key technical inhibitor barrier was solved.

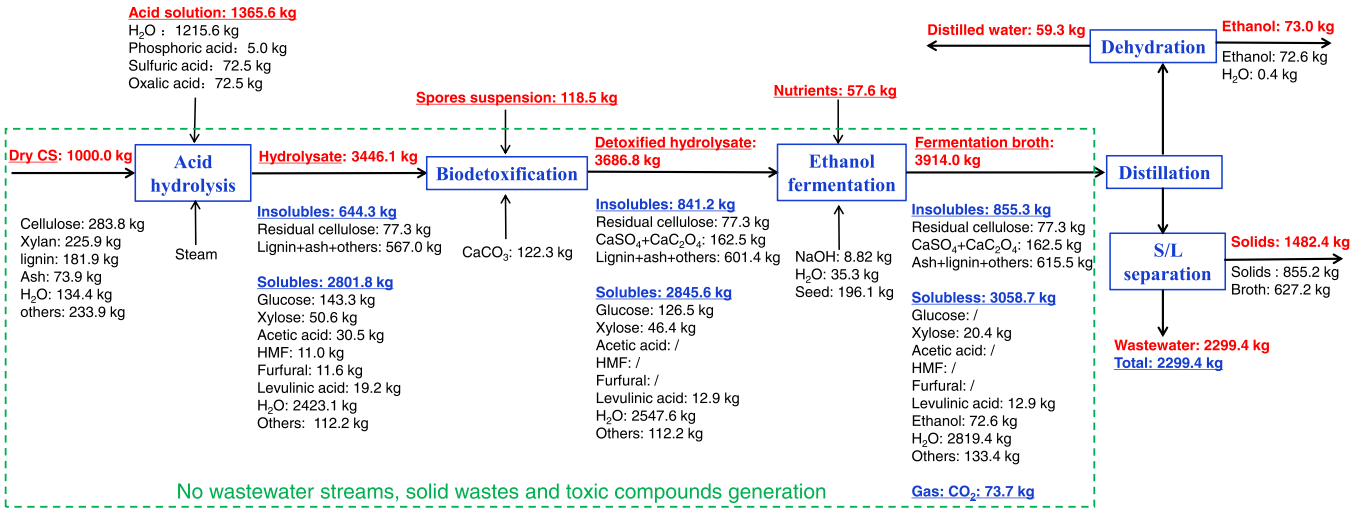

Fig. 2. Overall mass balance of mixed acid hydrolysis and cellulosic ethanol production from 1000.0 kg dry corn stover. The ethanol distillation was conducted in the bench-scale system equipped with a heat exchanger and spiral condenser. The ethanol concentration in the distillation of the glass was 54.9% (w/w). The aqueous solution of ethanol obtained by distillation needs to be further dehydrated to 99.5% (w/w) ethanol product.

Table 4
Process parameters and performances of dilute acid hydrolysis and enzymatic hydrolysis for cellulosic ethanol production.

	Case 1[a]	Case 2[b]	Case 3[c]	Case 4[c]
Processing	Direct acid hydrolysis	NREL process	Dry biorefinery	Dry biorefinery
Pretreatment or hydrolysis	Acid hydrolysis	Dilute acid pretreatment	Dilute acid pretreatment	Dilute acid pretreatment
Acid dosage (% DM)	7.25%SA+ 7.25%OA+ 0.50%PA	2.10% SA	2.50% SA	2.50% SA
S/L ratio	2:3	3:7	2:1	2:1
Temperature (°C)	185	158	175	175
Reaction time (min)	15 + 5[b]	5	5	5
Final solids content	19.8%	> 20.0%	45.2%	45.2%
Detoxification	Biodetoxification	Alkaline detoxification	Biodetoxification	Biodetoxification
Fermentation[d]	SHF	SHF	SSF	SSF
Initial solids loading	19.8% (w/w)	20.0% (w/w)	30.0% (w/w)	30.0% (w/w)
(Pre-) Hydrolysis	/	48 °C for 84 h	50 °C for 12 h	50 °C for 20 h
Cellulase	/	On-site	Purchase	Purchase
Cellulase dosage	/	25 mg/g cellulose	10 mg/g cellulose	5 mg/g cellulose
Ethanol titer	24.2 g/L (3.1%, v/v)	53.4 g/L (6.7%, v/v)	85.1 g/L (10.8%, v/v)	64.9 g/L (8.2%, v/v)
Ethanol yield (kg/ton CS)[e]	72.6	244.8	214.2	174.6

[a] Refers to Table 1, the parameters on the last case.
[b] Refers to NREL process (Humbird et al., 2011). Ammonia is used for alkaline detoxification.
[c] Refers to dry biorefinery process (Liu and Bao, 2017; Liu et al., 2018). Where Case 4 was obtained by reducing the cellulase dosage from 10 to 5 mg/g cellulose on the basis of Case 3.
[d] SHF, separate hydrolysis and fermentation; SSF, simultaneous saccharification and co-fermentation.
[e] The ethanol yield (kg/ton CS) was calculated according to the components of raw corn stover in this study.

3.4. Discussion

The primary challenge for dilute acid hydrolysis is how to raise glucose yield to an economically viable process (Shuai and Pan, 2012). Despite greater efforts had been made on substrate selection, continuous reactor use, hydrolysis kinetics control and glucose decomposition inhibition (Franzidis et al., 1982; Gurgel et al., 2012; Heinonen et al., 2012), the yield of glucose from cellulose is usually between 40% and 60% of the theoretical values because of the over-degradation of sugars from acid hydrolysis (Taherzadeh and Karimi, 2007). Unlike cellulase enzyme, the strong mineral or organic acids further dehydrate the obtained monosaccharides from polysaccharides hydrolysis into furan aldehydes (Gurgel et al., 2012; Lenihan et al., 2011), especially for xylose sugar (Table 1). To avoid the excessive loss of xylose at high temperature, the dilute acid hydrolysis could be carried out in two (or more) stages with low solids loading (Nagle et al., 1999; Sanchez et al., 2004), in which the hemicellulose is hydrolyzed to monosaccharides (xylose) under relatively mild severity in the first stage, while the residue solid is hydrolyzed in the second stage with higher severity. To process the "two stage" acid hydrolysis, complicated concentrating, detoxification, neutralization operations with wastewater and energy input are inevitable.

The second obstacle of acid hydrolysis process is the low concentrations of fermentable sugars in acid hydrolysates resulting the low ethanol titer in the consequent fermentation. Energy consumption of ethanol distillation sharply increases if the ethanol titer of the fermentation broth is below 4% (w/w) (Galbe et al., 2007; Zhao et al., 2020). Increasing ethanol titer requires the higher titers of fermentable sugars in the acid hydrolysate (minimum to 80–100 g/L), and this is a difficult target for acid hydrolysis (pre-concentrating by "evaporation" requires the same or even high energy consumption input (Zacchi and Axelsson, 1989)). This study conducted the one-stage acid hydrolysis at the high solids loading to guarantee a relatively high fermentable sugars concentration, but still below the economic level of sugars concentration. Obtaining highly titer of fermentable sugars with high yield is still a big challenge for acid hydrolysis process.

In conclusion, the general acid hydrolysis catalyzed by common organic or inorganic acids (or mixed acids) is not suitable for the current

Table 5
Materials balance and preliminary economic estimations of different biorefinery processing.

	Case 1	Case 2	Case 3	Case 4
Processing	Dilute acid hydrolysis	NREL process	Dry biorefinery	Dry biorefinery
(a) Materials balance				
Raw corn stover (ton/year)	300,000	300,000	300,000	300,000
Acid catalyst (ton/year)	45,000	6300	7500	7500
Cellulase (ton protein/year)[a]	/	1913	1186	533
Ethanol production (million gallons/year)	7.19	24.25	21.22	17.29
(b) Materials cost and ethanol sale				
Feedstock cost ($ million/year)[b]	17.73	17.73	17.73	17.73
Acid cost ($ million/year)[c]	10.30	0.82	0.98	0.98
Cellulase cost ($ million/year)	/	8.11[d]	7.43/27.62[e]	3.35/12.43[e]
Ethanol sale ($ million/year)[f]	15.89	53.60	46.89	38.22

[a] The annual cellulase usage was calculated according to the components of corn stover in this study.
[b] The price of corn stover is $ 59.0/ton according to the price offered by the local suppliers in Henan province, China.
[c] Phosphoric acid is neutralized to phosphate as nutrients for the subsequent ethanol fermentation. The cost of phosphoric acid is not included in the total cost of the acid catalysts.
[d] Assuming the cellulase is produced on-site with glucose as carbon source.
[e] Assume that the cellulase enzyme is purchased from Novozymes with the claimed enzyme cost of $0.50/gal ethanol. In the proposed process (Humbird et al., 2011), the ethanol production is 21,672.41 kg/h and the enzyme usage is 579 kg protein/h, which are equivalent to 7,256.35 Gal ethanol/h (21,672.41 kg/h is divided by the ethanol density 0.789 then transformed to gal). The enzyme protein price is equivalent to 7256.35 0.50/579 = $6.27/kg (2010 $). Assume that the cellulase enzyme is Youtell #6 purchased from Chinese enzyme market at 13 Chinese Yuan (RMB)/kg enzyme with the protein content of 9% (Fang et al., 2014), equivalent to $23.30/kg (2013$).
[f] The current ethanol sale price is $2.21/gal (https://tradingeconomics.com/commodity/ethanol).

biorefinery process compared to enzymatic hydrolysis. The future challenge on acid hydrolysis of lignocellulose is the development of novel cellulase-mimetic solid catalyst with high selectivity and activity of fermentable sugars without degradation of sugars to furan aldehydes (Huang and Fu, 2013; Shuai and Pan, 2012; Zhang et al., 2020). With the substantial breakthrough in highly active and selective acid catalyst, the dilute acid hydrolysis is expected to be competitive with enzymic hydrolysis and feasible for practical applications.

4. Conclusion

The parameters of dilute acid hydrolysis of corn stover were finally optimized to obtain 51.1 ± 1.7 g/L of glucose from cellulose (0.50 g/g cellulose) and 18.1 ± 0.7 g/L of xylose from xylan. The semi-continuous submerged biodetoxification by *P. variotii* FN89 completely removed furfural, HMF and acetic acid from corn stover acid hydrolysate with only 11.6% loss of the total fermentable sugars. The successful ethanol fermentation was conducted to obtain 24.2 ± 0.3 g/L of ethanol with the yield of 72.6 kg per metric ton of dry corn stover. The preliminary techno-economic evaluation based on the full-scale acid hydrolysis process shows that the ethanol production by the current dilute acid hydrolysis process was only 29.6–41.6% of that by three different enzymatic hydrolysis processes. The cost reduction with cheap acid catalysts, instead of cellulase either by producing on-site or purchasing, only compensated 21.5–89.1% of the ethanol production loss. The re-examination of acid hydrolysis process reveals that the future of acid hydrolysis depends on the development of novel cellulase-mimetic catalyst with high selectivity of fermentable sugars.

CRediT authorship contribution statement

Jie Bao: Conceptualization, Supervision, Funding acquisition, Writing – review & editing. **Bin Zhang:** Conceptualization, Data curation, Investigation, Writing – original draft. **Lei Wu:** Investigation, Methodology. **Ya Wang:** Investigation, Software. **Jing Li:** Investigation. **Baorui Zhan:** Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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