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ORIGINAL ARTICLE

Itaconic acid fermentation using activated charcoal-treated corn stover hydrolysate and process evaluation based on Aspen plus model

Yunlong Liu[1] · Gang Liu[1] · Jian Zhang[1] · Venkatesh Balan[2] · Jie Bao[1]

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Abstract
Itaconic acid production using lignocellulose materials is a promising approach to replace sugar substrates such as glucose that are expensive. However, the complicated detoxification of the hydrolysate is pre-requisite to remove the lignocellulose-derived inhibitors to enable high organic acid fermentation. In this study, the hydrolysate prepared using dry acid pretreated and biodetoxified corn stover was tested for itaconic acid production by the fungal strain *Aspergillus terreus* M69. Corn stover hydrolysate containing 0.85 g/L acetic acid severely inhibited the organic acid production, but a treatment on the hydrolysate with activated charcoal to remove partial acetic acid helped to produce 33.6 g/L itaconic acid at a yield of 0.56 g/g. Most acetic acid released during enzymatic hydrolysis other than pretreatment was responsible for the inhibition of itaconic acid production. The technol-economic analysis showed that the minimum itaconic acid selling price was $1.647 per kg, which was lower than its market price. This study demonstrates the great potential of itaconic acid production using lignocellulose.

Keywords Itaconic acid fermentation · Acetic acid · Lignocellulose · Dry acid pretreatment · Biodetoxification · Techno-economic analysis

1 Introduction

Itaconic acid belongs to one of the 12 most promising building block chemicals that could be produced from biomass [1]. With broad applications in synthesis of resins, coatings, and polymers, the worldwide itaconic acid production will reach 407,790 metric tons by 2020 with a total value of US$567 million [2]. As the cost of the carbon source (glucose) accounts for more than 25% of the total itaconic acid production cost in industry, using the cheap and low-cost lignocellulosic feedstock to produce itaconic acid may promote the bio-based technology and help to reduce the processing cost [3].

However, there were only a few reports available in literature on itaconic acid production by *Aspergillus terreus* strains using lignocellulosic biomass. Tippkotter et al. [4] reported that *A. terreus* NRRL 1960 could not grow on untreated organosolv beech wood hydrolysate due to the presence of phenolic inhibitors. After a complicated detoxification using NaOH washing and zeolite treatment to remove the phenolic compounds, as well as anion and cation exchanging to remove metal ions, 7.2 g/L itaconic acid was produced with a yield of 0.3 g/g based on the initial 20.5 g/L glucose and 4.0 g/L xylose. In another study carried out by Jimenz-Quero et al. [5], they were unsuccessful in fermenting itaconic acid using the hydrolysate of corncob and wheat bran even after 10-fold dilution. Li et al. [6] obtained 19.3 g/L itaconic acid from the undetoxified enzymatic hydrolysate of 10% (*w*/*w*) steam-exploded corn stover using an inhibitor-tolerant *A. terreus* mutant strain. Pedroso et al. [7] obtained 1.9 g/L itaconic acid

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✉ Jian Zhang
jzhang@ecust.edu.cn

[1] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China

[2] Department of Engineering Technology, Biotechnology Division, College of Technology, University of Houston, 4800 Calhoun Road, Houston, TX 77004, USA

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from the CaO-detoxified hydrolysate of phosphoric acid-pretreated rice husks. Krull et al. [8] obtained 27.7 g/L itaconic acid using the wheat chaff (hull) hydrolysate after purification by a cation exchange chromatography. It is evident that a complicated detoxification on the lignocellulose hydrolysate is pre-requisite for itaconic acid fermentation, which would lead to massive amount of wastewater generation/fermentable sugar loss, resulting in high cost of production.

In our previous studies, dry acid-pretreated biomass was subjected to biodetoxification using fungus *Amorphotheca resinae* ZN1 at high solid loading (40–50%, *w*/*w*). During solid-state biodetoxification, the fungus *A. resinae* ZN1 was able to degrade almost all the inhibitory compounds generated in acid pretreatment efficiently without wastewater generation and using low energy input [9–12]. This comprehensive process (dry acid pretreatment followed by biodetoxification) has demonstrated to be effective in producing cellulosic biofuels and biochemical [9, 10, 13–15]. In this study, we tested the inhibitors tolerance of the itaconic acid-producing strain *A. terreus* M69 and demonstrated the potential of itaconic acid production using the enzymatic hydrolysate of corn stover processed by dry acid pretreatment and biodetoxification. In addition, a preliminary economic analysis was conducted to evaluate the cellulosic itaconic acid production process.

2 Materials and methods

2.1 Raw materials

The corn stover was harvested in fall 2016 from Tongliao, Inner Mongolia, China. After the corn stover was shipped to Shanghai, it was milled to 10 mm in diameter in a beater pulverizer before use. The compositions of raw corn stover were 33.0% glucan, 26.9% xylan, 20.8% lignin, and 6.3% ash (dry weight basis) [16].

2.2 Enzymes and strains

The enzyme Cellic CTec2 (Novozymes, Tianjin, China, batch No. VSC10008) with filter paper activity of 203.2 FPU/mL and cellobiase activity of 4900 CBU/mL was used in this study [17, 18]. The protein content of Cellic CTec2 was 87.3 mg/mL analyzed using Bradford method [19].

Amorphotheca resinae ZN1 (CGMCC 7452, China General Microbial Collection Center, Beijing, China) was used to degrade inhibitors generated during dry acid pretreatment by solid-state biodetoxification [11]. The fungal strain *Aspergillus terreus* M69 purchased from Shanghai Industrial Microbiology Institute (SIIM, Shanghai, China) was used for itaconic acid fermentation.

2.3 Pretreatment and biodetoxification

The dry acid pretreatment was used to pretreat the corn stover [12, 20]. The corn stover and a sulfuric acid solution (7.6%, *w*/*w*) were concurrently fed into the helical-stirring pretreatment reactor (pCF20-16, Keli Chemical Equipment, Yantai, China) at a solid/liquid ratio of 2:1 (*w*/*w*) and maintained at 175 °C for 5 min with an agitation speed of 50 rpm. The solid content of the pretreated corn stover was around 50% (*w*/*w*). No liquid stream was produced during this process.

Biodetoxification via solid-state fermentation using *A. resinae* ZN1 strain was used to detoxify the pretreated corn stover [9, 11]. Firstly, 20% (*w*/*w*) $Ca(OH)_2$ slurry was used to neutralize the acidic pretreated corn stover to pH 5.5. Secondly, the neutralized corn stover was disk milled to reduce the large cellulose particles before inoculating the *A. resinae* ZN1 seeds at 10% (*w*/*w*) ratio. The preparation of *A. resinae* ZN1 seeds was as follows: *A. resinae* ZN1 was stored and transferred on a potato dextrose agar (PDA) medium slant and cultured at 28 °C for 5–7 days. The slant (including PDA medium and *A. resinae* ZN1) was inoculated onto 200-g pretreated and neutralized corn stover and cultivated at 28 °C in a sealed plastic box for 5 days. Then, the cultured *A. resinae* ZN1 on solid corn stover was used as biodetoxification seeds [9, 11]. The biodetoxification was conducted for 72 h in a 15-L helical-stirring bioreactor (Biotech-15JGZ, Shanghai Baoxing Biotech Equipment CO., Shanghai, China) at 1.0 vvm of aeration with a periodical agitation (50 rpm for 60 s every 12 h).

2.4 Corn stover hydrolysate preparation and activated charcoal treatment

The enzymatic hydrolysis of pretreated and biodetoxified corn stover was conducted at 15% (*w*/*w*) solid loading, 4 mg cellulase protein/g dry matter (equal to 9.3 FPU/g dry matter), 50 °C in a 5-L helical stirring bioreactor for 48 h. The pH was maintained at 4.8 automatically with NaOH solution (20%, *w*/*w*). The hydrolyzed slurry was solid-liquid separated by centrifugation at 10,000 rpm (12,096×*g*) for 10 min. The clear corn stover hydrolysate containing 70.65 g/L glucose, 8.58 g/L xylose, and 0.85 g/L acetic acid was used for fermentation. Furfural, HMF, and phenolic acids were below the detection limit.

A portion of corn stover hydrolysate was detoxified using activated charcoal (200 mesh, Sinopharm Chemical Reagent Co., Shanghai, China). The pH of the hydrolysate was firstly adjusted to 2.5 using H_2SO_4 (10 M); then, the treatment was conducted at 30 °C, 180 rpm for 90 min with 10% (*w*/*v*) activated charcoal [21]. The solid-liquid separation was carried out by filtration to remove the activated charcoal. The detoxified corn stover hydrolysate contained 64.30 g/L glucose, 6.74 g/L xylose, and 0.45 g/L acetic acid.

2.5 Itaconic acid fermentation

The spore suspension of *A. terreus* M69 was transferred onto potato-dextrose-agar (PDA) Petri dish plates and cultured at 28 °C for 4–6 days. Then, the spores were washed by 0.75% (*w*/*w*) saline solution and inoculated into a 500-mL flask containing 100-mL seed medium at about 3.0×10^6 per mL for seed culturing at 35 °C, 200 rpm. After 24-h cultivation, the seed broth was transferred into a 250-mL flask containing 50-mL fermentation medium with 10% (*v*/*v*) inoculation ratio at 35 °C, 250 rpm for 120 h. For the inhibitor tolerance assays, the acetic acid, formic acid, furfural, HMF, syringaldehyde, vanillin, or 4-hydroxybenzaldehyde was supplemented to the fermentation media to a required concentration individually. Itaconic acid production medium based on corn stover hydrolysate was the same with the composition of fermentation medium indicated below except glucose. Samples were taken periodically for high-performance liquid chromatography (HPLC) analysis. All the fermentation was carried out in duplicate. Two different media used were shown below:

(1) Seed medium: 65 g glucose, 3.3 g $(NH_4)_2SO_4$, 0.8 g $MgSO_4 \cdot 7H_2O$, 1 g itaconic acid, 0.088 g KH_2PO_4, 0.004 g $CuSO_4 \cdot 5H_2O$ in 1 L of deionized water at pH 2.8 [22].
(2) Fermentation medium: 65 g glucose, 1.534 g $(NH_4)_2SO_4$, 0.11 g KH_2PO_4, 2.08 g $MgSO_4 \cdot 7H_2O$, 1 g $CaCl_2 \cdot 2H_2O$, 0.074 g NaCl, 0.2 mg $CuSO_4 \cdot 5H_2O$, 5.5 mg $FeSO_4 \cdot 7H_2O$, 0.7 mg $MnCl_2 \cdot 4H_2O$, 1.3 mg $ZnSO_4 \cdot 7H_2O$, in 1 L of deionized water at pH 3.1 [23].

2.6 HPLC analysis

Glucose, xylose, formic acid, acetic acid, furfural, HMF, vanillin, syringaldehyde, and 4-hydroxybenzaldehyde were analyzed by HPLC [9, 24]. Itaconic acid was determined using HPLC (LC-20AT, UV/VIS detector SPD-20A, Shimadzu, Kyoto, Japan) fitted with a Bio-Rad Aminex HPX-87H column at 55 °C using 5 mM H_2SO_4 as eluent at the rate of 0.6 mL/min and the detection wavelength of 210 nm.

2.7 Calculation

The calculation of itaconic acid yield was based on the ratio of produced itaconic acid to the total glucose at the beginning of the fermentation as follows:

$$\text{Itaconic acid yield} = \frac{[IA] \times V - [IA]_0 \times V_0}{[Glu] \times V_0}$$

where $[IA]_0$ and $[IA]$ were itaconic acid concentrations at the beginning and the end of fermentation (g/L), respectively; V_0 and V were the volume at the beginning and the end of fermentation (L), respectively; $[Glu]$ was the glucose concentration at the beginning of the fermentation.

2.8 Process model establishment

The Aspen plus simulation was preformed based on related experiment results and the cellulosic ethanol model of NREL report [25], including ten process areas of feedstock handing (A100), pretreatment (A200), detoxification (A250), hydrolysis and fermentation (A300), cellulase enzyme production (A400), product recovery (A500), wastewater treatment (A600), storage (A700), combustor-boiler-turbogenerator (A800), and utilities (A900) shown in Fig. 1 [25]. The biorefinery plant handles with 300,000 metric tons of corn stover feedstock annually with an annual operation time of 8000 h. The main input data for the established model is shown in Table S1. The major differences compared with the cellulosic ethanol model included [15]

(1) In the hydrolysis and fermentation area (A300), the solid loading of corn stover was 15% (*w*/*w*). Prior to fermentation, the enzymatic hydrolysate was detoxified using 2% activated charcoal to remove partial acetic acid [26].
(2) In the product recovery area (A500), the operations of two evaporation and crystallization, decoloration, final crystallization, and drying were conducted sequentially to obtain itaconic acid product with a purity of 99% [27, 28].

2.9 Techno-economic analysis

The technical economic analysis of cellulosic itaconic acid production from corn stover was carried out based on the assumption of "n*th*-plant" and the reference year of 2013 [25]. The exchange rate of 1:6.2 from US dollar ($) to Chinese Yuan (CNY) was set (http://data.stats.gov.cn/). The prices of the main equipment and raw material shown in Table S2 and Table S3 were quoted by the related Chinese corporations [29]. The mass and energy balance data, operating costs, and total capital investment were calculated according to the Aspen plus simulation, total equipment cost, and plant capacity, respectively. With these costs, a discounted cash flow rate of return analysis to determine the minimum itaconic acid selling price (MIASP, $/gal) required to obtain a net present value of zero with 10% internal rate of return after taxes. The parameters in the economic analysis are listed in Table S4.

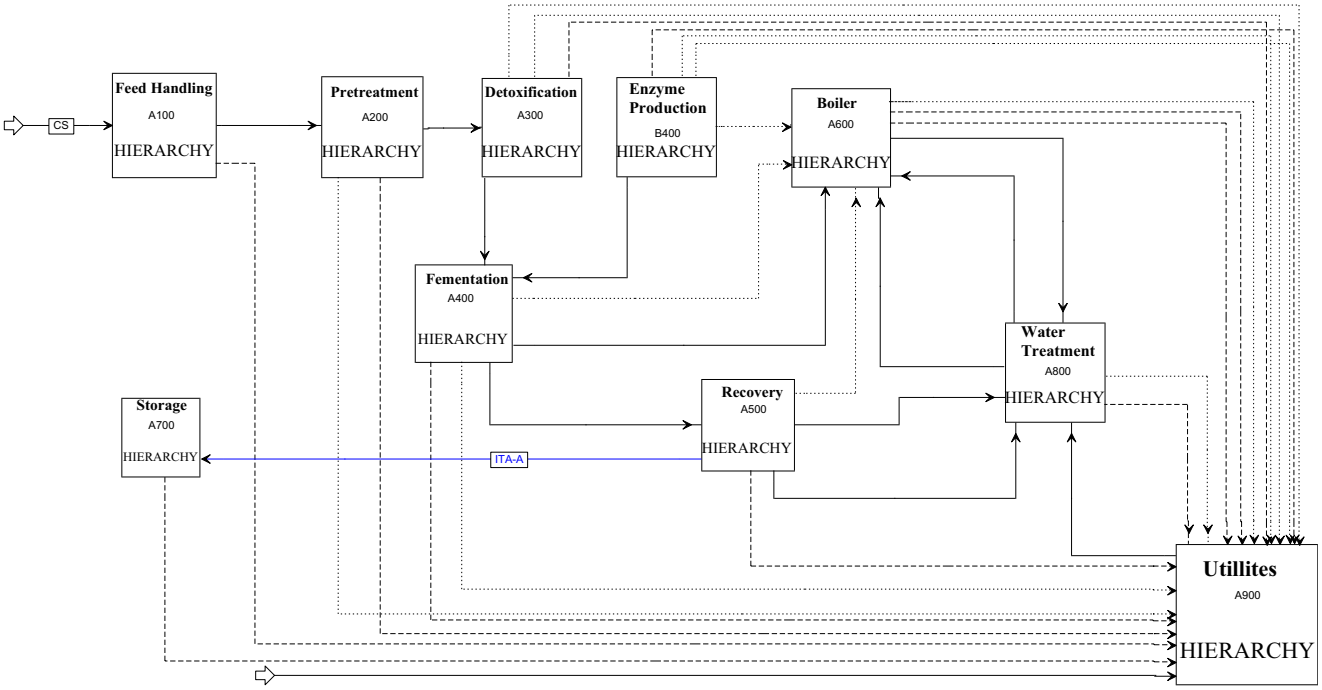

Fig. 1 General flowsheet of cellulosic itaconic acid production using corn stover processed by dry acid pretreatment and biodetoxification

3 Results and discussion

3.1 Effect of lignocellulose-derived inhibitors on cell growth and itaconic acid production

Weak organic acids including acetic acid and formic acid, furan derivatives including furfural and HMF, and lignin derivatives including syringaldehyde, vanillin and 4-hydroxybenzaldehyde are usually generated during lignocellulose pretreatment under harsh pretreatment conditions [9, 11, 12]. The effect of these typical lignocellulose-derived inhibitors on both cell growth and itaconic acid production of *A. terreus* M69 was tested (shown in Fig. 2). There was no itaconic acid production in the presence of 1.0 g/L acetic acid. However, the fungal biomass of *A. terreus* M69 was found to be still active while the morphology of the fungus changed from small homogenous pellets to a complete large pellet with the increasing acetic acid concentrations. In contrast, Saha et al. [30] did not observe any cell growth and itaconic acid in the presence of 0.8 g/L acetic acid. Formic acid showed more toxicity to *A. terreus* M69. Almost no itaconic acid was detected when furfural concentration was higher than 0.50 g/L in the fermentation medium. However, HMF was found to be less toxic. There was no itaconic acid generation when its concentration was higher than 0.75 g/L for vanillin, or higher than 1.0 g/L for 4-hydroxybenzaldehyde, respectively. Syringaldehyde exhibited the weakest inhibition among the three phenolic compounds.

From these studies, it is clear that similar to ethanol and lactic acid producing microorganisms, itaconic acid producing strain *A. terreus* is also sensitive to the lignocellulose-derived inhibitors [31, 32]. These inhibitory compounds should be removed or maintained at a low level in the hydrolysate in order to get higher titers of itaconic acid using the strain *A. terreus.*

3.2 Acetic acid released during enzymatic hydrolysis impeded the itaconic acid production

The weak acids, furan derivatives, and phenols in the pretreated corn stover solids are almost undetectable by HPLC after biodetoxification using fungus *A. resinae* ZN1 [11]. In the hydrolysate after enzymatic hydrolysis of the pretreated and biodetoxified corn stover, other inhibitors were undetectable besides 0.85 g/L acetic acid. This amount of acetic acid possibly came from auto-hydrolysis of acetyl groups in the corn stover during enzymatic hydrolysis stage. Figure 3 shows that no itaconic acid could be produced by *A. terreus* M69 using the corn stover hydrolysate. As no other inhibitors were detected, 0.85 g/L acetic acid was considered as the main factor that inhibited itaconic acid production. The acetic acid concentration was reduced to 0.45 g/L after treatment with activated charcoal accompanied with small amount of sugar loss. Figure 3 also shows that 33.6 g/L itaconic acid with a yield of 0.56 g/g was obtained using the corn stover hydrolysate treated with activated charcoal, which was close to the fermentation performance using synthetic medium. In addition, when the acetic acid was supplemented back to the activated charcoal-treated hydrolysate to ~0.9 g/L, the

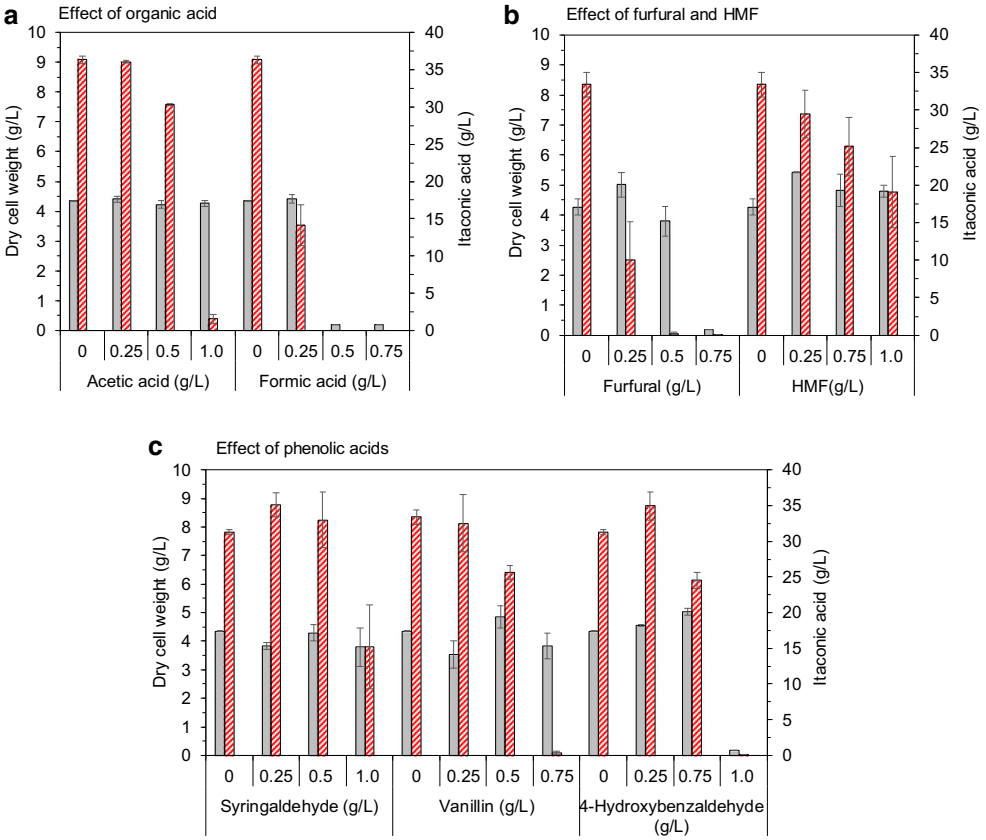

Fig. 2 Effect of lignocellulose derived inhibitors on the growth of *A. terreus* M69 (gray bar) and itaconic acid production (red bar). Here, **a** effect of weak acids, **b** effect of furfural and HMF, and **c** effect of phenolic acids. The fermentation was performed in duplicate in shake flasks at 35 °C, 250 rpm for 120 h

itaconic acid fermentability was lost again. The results in Fig. 3c show that the fungal strain *A. terreus* could degrade acetic acid, but there was still no itaconic acid generation when the acetic acid was decreased below the inhibition level. These results implied that > 0.8 g/L acetic acid in the corn stover hydrolysate should be responsible for the total inhibition of itaconic acid production. Among the reported itaconic acid fermentation using lignocellulosic materials shown in Table 1, 33.6 g/L itaconic acid with a yield of 0.56 g/g obtained in this study is the highest.

The un-dissociated acetic acid could cross the plasma membrane into the cytosol, cause the drop of intracellular pH, and lead to low enzyme activity in the cell or even the cell death [33]. In addition, the low fermentation pH 3.1 facilitates the formation of un-dissociated acetic acid and enhances its inhibition effect [8]. When the fermentation pH was adjusted to 5.0–6.0, the fungus *A. terreus* M69 grew well, but no itaconic acid could be produced (data were not shown). The itaconic acid production requires a low pH environment, but it is difficult to reduce the pH of the hydrolysate from 5.0–6.0 to

Table 1 Comparison of itaconic acid production using lignocellulosic biomass

Feedstock	Pretreatment	Detoxification of the enzymatic hydrolysate	Itaconic acid (g/L)	Yield (g/g)	References
Beech wood	Organosolv pretreatment and alkali washing	Ion exchanging	7.2	0.3 [a]	[4]
Corncobs	Dilute acid pretreatment	No	0	0	[5]
Corn stover	Steam explosion and water washing	No	19.3	0.36 [b]	[6]
Rice husks	Phosphoric acid pretreatment	CaO	1.9	0.049 [b]	[7]
Wheat chaff	Alkaline pretreatment and water washing	Ion exchanging	27.7	0.41 [c]	[8]
Corn stover	Dry acid pretreatment and biodetoxification	No	0	0	This study
Corn stover	Dry acid pretreatment and biodetoxification	Activated charcoal treatment	33.6	0.56	This study

[a] The calculation of itaconic acid yield is based on the sugars in the hydrolysate

[b] The calculation of itaconic acid yield is based on the consumed glucose in the hydrolysate

[c] The calculation of itaconic acid yield is based on the consumed total sugars in the hydrolysate

Table 2 Impact of detoxification and xylose utilization on minimum itaconic acid selling price (MIASP) calculated based on the Aspen plus modeling

MIASP ($/kg)	Base case[a]	Case 1[b]	Case 2[c]
Itaconic acid yield from glucose (g/g)	0.54	0.54	0.54
Itaconic acid yield from xylose (g/g)	0	0	0.40
Detoxification with activated charcoal	Yes	N/A	N/A
Itaconic acid yield (kg/ton corn stover)	151	163	220
MIASP ($/kg)	1.647	1.143	0.875
Feedstock cost ($/kg)	0.408	0.378	0.280
Enzyme cost ($/kg)	0.208	0.192	0.143
Conversion cost ($/kg)	1.031	0.573	0.452

[a] The calculation was based on this study

[b] The calculation assumed that the fermenting strain was inhibitor tolerant, and the treatment with activated charcoal could be deleted

[c] The calculation assumed that the treatment with activated charcoal could be deleted, and the fermenting strain could co-ferment glucose and xylose into itaconic acid [28]

2.0–3.0 due to the buffering effect of acetate present in the hydrolysate [22, 34].

As dilute acid pretreatment and biodetoxification could not remove all the acetyl groups that are present in hemicellulose, some of the left over acetyl groups are hydrolyzed during enzymatic hydrolysis step. Hence, the hydrolysate has to be detoxified to decrease the acetic acid concentration and ensure the fermentability of substrate. De-acetylation and mechanical refining (DMR) processing reported by NREL, aiming to remove acetyl groups completely before enzymatic hydrolysis, might be a suitable option for cellulosic itaconic acid production [35]. On the other hand, evolutionary adaptation of *A. terreus* is an alternative strategy to obtain the acetic acid tolerant strains.

3.3 Techno-economic analysis of cellulosic itaconic acid production

The techno-economic evaluation was carried out using the maximum itaconic acid concentration obtained in this study (base case with itaconic acid titer of 33.6 g/L). Table 2 shows that the minimum itaconic acid selling price (MIASP) was $1.647 per kg (base case). The cost of feedstock, enzyme, and non-enzyme conversion were $0.408, $0.208, and $1.031 per kg, respectively. This MIASP value was a slight

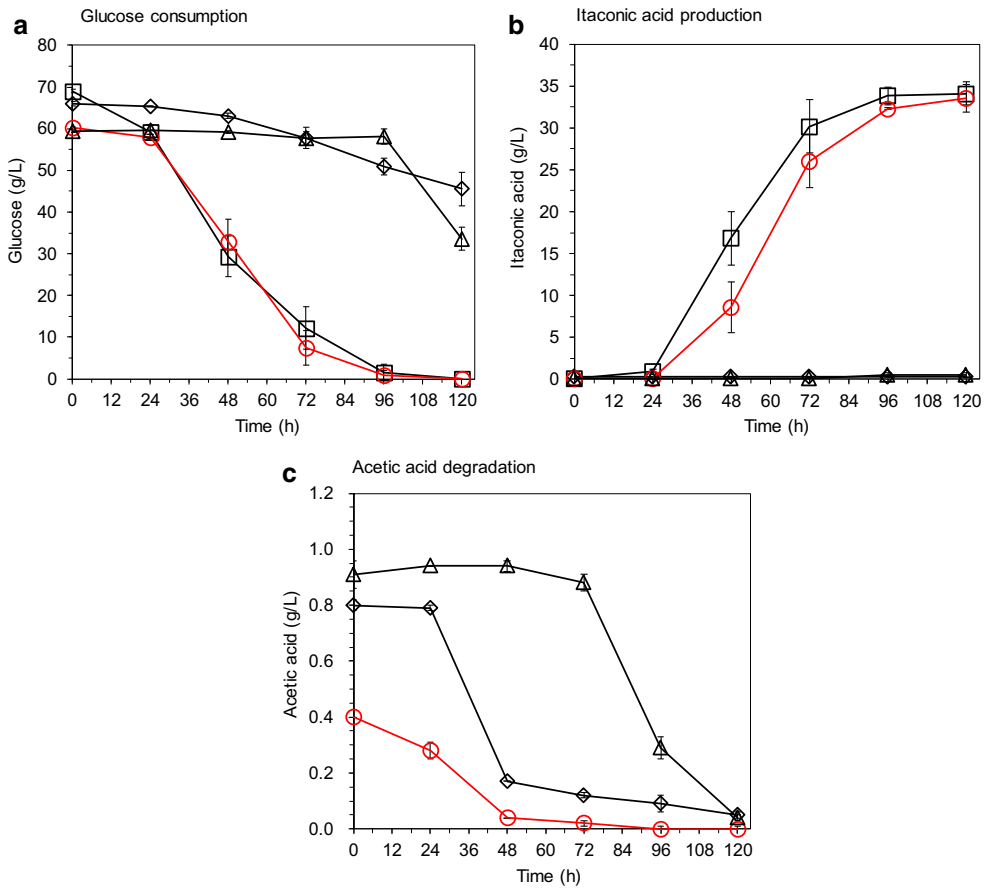

Fig. 3 Itaconic acid fermentation using synthetic fermentation medium (black square), crude corn stover hydrolysate (black diamond), detoxified corn stover hydrolysate with activated charcoal (red circle), and detoxified hydrolysate with acetic acid addition (black triangle). Here, **a** glucose consumption, **b** itaconic acid production, and **c** acetic acid degradation. All the fermentation experiment was carried out in duplicate in shake flasks at 35 °C, 250 rpm for 120 h

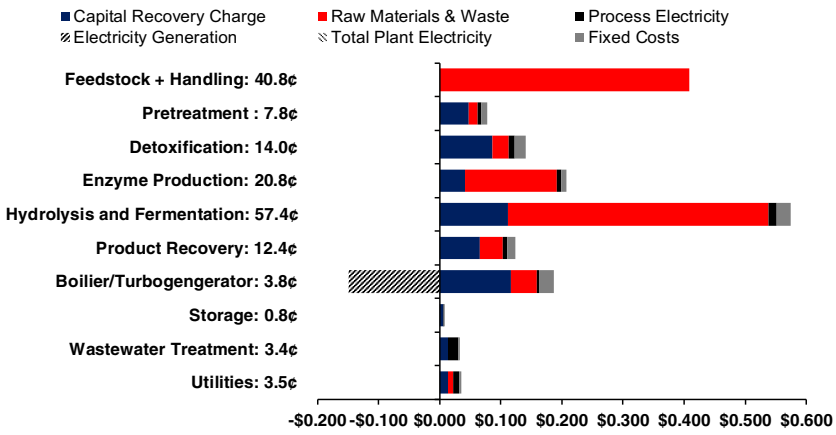

Fig. 4 Cost contribution details from each process area (per kg itaconic acid) in the base case

lower than the market price of itaconic acid ($1.8 per kg) according to Nieder-Heitmann et al. [28].

For the base case, the cost of hydrolysis and fermentation unit was $0.574 for 1-kg itaconic acid production (shown in Fig. 4), whereas the cost of activated charcoal (used for acetic acid removal) accounted for 71% of this unit. Besides the high activated charcoal cost, hydrolysate detoxification also resulted in fermentable sugar loss and itaconic acid yield decrease. Therefore, case 1 assumes that the detoxification with activated charcoal could be deleted with the evolution of the fermenting strains to tolerate ~ 1 g/L acetic acid. The itaconic acid yield was increased to 163 kg per ton corn stover due to no sugar loss without detoxification, and the MIASP was decreased to $1.143 per kg. Furthermore, case 2 shows the potential of co-fermentation of glucose and xylose into itaconic acid. The itaconic acid yield could reach 220 kg per ton corn stover, while the MIASP could be reduced to $0.875 per kg. Evidently, enabling the fermenting strains to overcome inhibition and co-fermentation of glucose and xylose would make the cellulosic itaconic acid production more cost-effective.

4 Conclusions

The itaconic acid producing fungal strain *A. terreus* M69 was sensitive to the lignocellulose-derived inhibitors. No itaconic acid was produced when dry acid pretreated and biodetoxified corn stover hydrolysate was used, while 33.6 g/L itaconic acid with a yield of 0.56 g/g was obtained after the hydrolysate was treated with activated charcoal. The acetic acid with a concentration higher than 0.8 g/L in the corn stover hydrolysate was responsible for the total inhibition of the *A. terreus* M69 strain. The minimum itaconic acid selling price (MIASP) was $1.647 per kg calculated based on the established Aspen plus model, which shows great potential for cellulosic itaconic acid production.

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