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Fermentative production of high titer citric acid from corn stover feedstock after dry dilute acid pretreatment and biodetoxification

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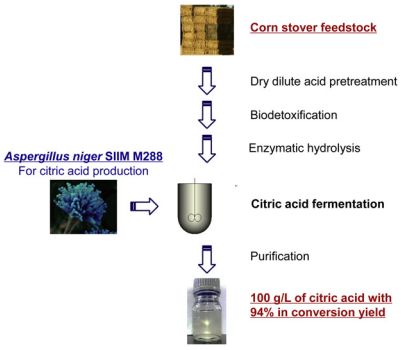
HIGHLIGHTS

- High citric acid accumulation was obtained from corn stover feedstock.
- 100.04 g/L of citric acid with yield of 94.11% were obtained from corn stover.
- No extra inorganic salts or inducers are required to corn stover hydrolysate.
- Zero waste water was generated from pretreatment to citric acid fermentation.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of this work is to study the citric acid fermentation by a robust strain *Aspergillus niger* SIIM M288 using corn stover feedstock after dry dilute sulfuric acid pretreatment and biodetoxification. Citric acid at 100.04 g/L with the yield of 94.11% was obtained, which are comparable to the starch or sucrose based citric acid fermentation. No free wastewater was generated in the overall process from the pretreatment to citric acid fermentation. Abundant divalent metal ions as well as high titer of potassium, phosphate, and nitrogen were found in corn stover hydrolysate. Further addition of extra nutrients showed no impact on increasing citric acid formation except minimum nitrogen source was required. Various fermentation parameters were tested and only minimum regulation was required during the fermentation. This study provided a biorefining process for citric acid fermentation from lignocellulose feedstock with the maximum citric acid titer and yield.

1. Introduction

Citric acid is the most widely used organic acid in food, beverage, pharmacy, cosmetics, agriculture, construction and chemical industries. The global production of citric acid has reached 1.6 million tons in 2007 with the increasing demand of 3.5–4.0% (Berovic and Legisa, 2007; Anastassiadis et al., 2008). Various cheaper or more available feedstocks such as brewer's grain waste, apple pomace, coffee husk, corn cobs, grape pomace, orange waste, pineapple waste on replacing starch or sucrose feedstocks on citric acid fermentation had been investigated (Schuster et al., 2002; Soccol et al., 2006; Angumeenal and Venkappayya, 2013).

Among these renewable resources, lignocellulose biomass is the most important option for its abundance and availability. However, utilizations of lignocellulose feedstock had frequently led to the very low citric acid titer as well as high waste water generations, thus the future of lignocellulose feedstock for citric acid production is uncertain. Hang and Woodams (1998) produced 250 g citric acid per kilogram corncob by solid state fermentation of *A. niger* NRRL

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2001 with less than 50% overall yield. Zhang et al. (2001) produce 10.52 g/L citric acid with the yield of 60.8% from wheat stalk by *A. niger* van Tieghem after complicated alkali and oxidative anthraquinone cooking before enzymatic hydrolysis and fermentation. Khosravi-Darani and Zoghi (2008) produced a relatively higher citric acid titer (34 g/L) with the yield of 34% by solid state fermentation of *A. niger* ATCC 9142 using pretreated sugarcane bagasse feedstock. There is still a large gap of citric acid titer between the current research achievements and the industrial applications for citric acid production from lignocellulose feedstock.

The purpose of this study is to increase the citric acid titer up to 100 g/L, which is considered as the minimum titer for practical citric acid fermentation, from lignocellulose feedstock while maintaining the high yield. Approaches of dry dilute acid pretreatment, biodetoxification and high solids content saccharification and fermentation (Zhang et al., 2010a,b, 2011; He et al., 2014a,b, 2016) had been successful applied on productions of high titer ethanol (Qureshi et al., 2015), lactic acid (Zhao et al., 2013; Liu et al., 2015), and gluconic acid (Zhang et al., 2016a,b). In this study, the biorefining methods are applied to citric acid fermentation from corn stover feedstock by a robust industrial strain *A. niger* SIIM M288. Citric acid titer of 100.04 g/L and the yield of 94.11% were obtained in fermentors and this is the maximum result of citric acid production from corn stover up to our knowledge. The result could be advanced into the prototype of commercial citric acid production from lignocellulose feedstock comparable to starch based fermentations.

2. Materials and methods

2.1. Raw materials

Corn stover used in flask fermentation was grown in Dancheng, Henan, China and harvested in fall 2012. Corn stover used in bioreactor fermentation was grown in Bayan Nur, Inner Mongolia, China and harvested in fall 2015. After collection, corn stover was milled coarsely using a beater pulverizer and screened through a mesh with the circle diameter of 10 mm. The milled corn stover was washed to remove the field dirt, stones and metals, then dried and stored in sealed plastic bags for use. The raw corn stover for flask fermentation contained 32.06% of cellulose, 20.55% of hemicellulose, 26.51% of lignin, 4.43% of ash (w/w), while the raw corn stover for bioreactor fermentation contained 35.72% of cellulose, 22.78% of hemicellulose, 15.68% of lignin, 3.59% of ash (w/w) determined by cellulose analyzer (Cellulose Analyzer 220, Ankom Technology, Macedon, NY, USA).

2.2. Enzymes and reagents

The commercial cellulase enzyme Youtell #6 was purchased from Hunan Youtell Biochemical Co., Yueyang, Hunan, China. The filter paper activity was 135 FPU per gram according to the protocol of NREL LAP-006 (Adney and Baker, 1996), the cellobiase activity was 344 CBU per gram enzyme reagent determined according to Ghose (1987), and the protein content was 90 mg per gram of enzyme determined by Bradford method (Bradford, 1976).

Acetic acid, formic acid, and levulinic acid were purchased from Sinopharm Chemical Reagent Co., Shanghai, China. Vanillin was purchased from Aladdin, Shanghai, China. Furfural and 5-hydroxymethylfurfural (HMF) were from J&K Scientific Co., Beijing, China. 4-Hydroxybenzaldehyde and syringaldehyde were from Sangon Biotech Co., Shanghai, China. All other chemicals and reagents including glucose, KH_2PO_4, NH_4Cl, $MgSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $FeSO_4{\cdot}7H_2O$, $MnCl_2$, NaOH, H_2SO_4, $Ca(OH)_2$, methanol and ethanol were analytical pure and obtained from Lingfeng Chemical Reagent Co., Shanghai, China.

2.3. Strains and media

A. niger SIIM M288 strains was purchased from Shanghai Industrial Institute of Microbiology (SIIM), Shanghai, China. The stock cultures of spores were maintained at −80 °C freezer in the synthetic medium containing 30% (v/v) glycerol solution.

Amorphotheca resinae ZN1 was isolated from our previous study and stored in China General Microorganism Collection (CGMCC #7452), Beijing, China. *A. resinae* ZN1 was used for the removal of inhibitors from the pretreated corn stover (Zhang et al., 2010b). Stock cultures and activation cultures were carried out in PDA medium at 4 °C and 28 °C, respectively.

The culture media used in this study included:

(1) PDA (potato-dextrose-agar) medium for culture of *A. resinae* ZN1 as well as *A. niger* SIIM M288 contained 200 g of potato extract juice, 20 g of glucose, 20 g of agar in 1 L of deionized water.
(2) Seed medium used for seed culture of *A. niger* SIIM M288 consisted of (g/L) glucose, 70; NH_4Cl, 2.5; KH_2PO_4, 2.5; $MgSO_4{\cdot}7H_2O$, 0.25; $CuSO_4{\cdot}5H_2O$, 2.36×10^{-4}; $ZnSO_4{\cdot}7H_2O$, 1.1×10^{-3}; $FeSO_4{\cdot}7H_2O$, 6.45×10^{-3}; and $MnCl_2$, 3.6×10^{-3}.
(3) Synthetic medium used for *A. niger* SIIM M288 fermentation consisted of (g/L) glucose, 100; NH_4Cl, 2.5; KH_2PO_4, 2.5; $MgSO_4{\cdot}7H_2O$, 0.25; $CuSO_4{\cdot}5H_2O$, 2.36×10^{-4}; $ZnSO_4{\cdot}7H_2O$, 1.1×10^{-3}; $FeSO_4{\cdot}7H_2O$, 6.45×10^{-3}; and $MnCl_2$, 3.6×10^{-3}.

2.4. Pretreatment and biodetoxification operations

Corn stover was pretreated in a 20 L reactor using dry dilute sulfuric acid pretreatment method according to Zhang et al. (2011) and He et al. (2014a,b). Briefly, milled corn stover materials and dilute sulfuric acid solution at 5.0% (w/w) were co-currently fed into the reactor at a solid/liquid ratio of 2:1 (w/w) with helically stirring at 50 rpm, then pretreated at 175 ± 1 °C for 5 min. The solids content of the pretreated slurry was around 50% (w/w) and no free wastewater stream was generated. The pretreated corn stover for flask fermentation contained 35.47% cellulose, 6.80% hemicellulose (w/w). The pretreated corn stover for bioreactor experiment contained 37.34% cellulose, 5.88% hemicellulose (w/w). Both were based on the dry matter weight and measured by two-step acid hydrolysis method according to NREL protocols (Sluiter et al., 2008).

Biodetoxification was carried out following the procedure in Zhang et al. (2010b) and He et al. (2016). Briefly, the pretreated corn stover was neutralized with 20% (w/w) $Ca(OH)_2$ suspension slurry to pH of 5–6, then spores of the biodetoxification fungus *A. resinae* ZN1 were inoculated onto the solids pretreated corn stover and lasted for 4–7 days at 28 °C until 90% of furfural and HMF were removed.

2.5. Hydrolysate and medium preparations

Dry dilute acid pretreated and biodetoxified corn stover was hydrolyzed using 15 FPU cellulase per gram of dry corn stover matter (DM) for 48 h at 50 °C. Then the water-insoluble solids were removed by centrifugation and the supernatant was used as the hydrolysate for citric acid fermentation after autoclaved at 115 °C for 20 min and filtered through filter paper. Three types of corn stover hydrolysate were prepared:

(1) High glucose hydrolysate for flask fermentations. Hydrolyzing 25% (w/w) of the pretreated and biodetoxified corn stover and the hydrolysate contained 104.50 g/L of glucose, 22.86 g/L of xylose, 3.50 g/L of acetic acid, 0.05 g/L of HMF (furfural was not detected) unless mentioned elsewhere. Corn stover hydrolysate using the freshly pretreated corn stover (not detoxified) was used as control contained 68.71 g/L of glucose, 34.10 g/L of xylose, 1.09 g/L of furfural, 0.84 g/L of HMF, 7.80 g/L of acetic acid, 2.46 g/L of formic acid, 1.43 g/L of levulinic acid, 0.43 g/L of vanillin, 0.20 g/L of syringaldehyde, and 0.02 g/L of 4-hydroxybenzaldehyde.
(2) Low glucose hydrolysate for flask fermentations. Hydrolyzing 15% (w/w) of the pretreated and biodetoxified corn stover and the hydrolysate contained 48.05 g/L of glucose, 6.54 g/L of xylose, 1.43 g/L of acetic acid (HMF and furfural were not detected).
(3) High glucose hydrolysate for bioreactor fermentations. Hydrolyzing 25% (w/w) of the pretreated and biodetoxified corn stover and the hydrolysate contained 99.67 g/L of glucose, 26.28 g/L of xylose, 1.74 g/L of acetic acid (furfural and HMF were not detected) unless mentioned elsewhere.

The inorganic salts were supplemented into the high glucose hydrolysate with (g/L) NH_4Cl, 2.5; KH_2PO_4, 2.5; $MgSO_4{\cdot}7H_2O$, 0.25; $CuSO_4{\cdot}5H_2O$, 2.36×10^{-4}; $ZnSO_4{\cdot}7H_2O$, 1.1×10^{-3}; $FeSO_4{\cdot}7H_2O$, 6.45×10^{-3}; and $MnCl_2$, 3.6×10^{-3}, same to the synthetic medium unless mentioned elsewhere.

The inducers were supplemented into the high glucose hydrolysate with the gradient 1%–5% (v/v), and the hydrolysate were supplemented (g/L) NH_4Cl, 2.5; KH_2PO_4, 2.5; $MgSO_4{\cdot}7H_2O$, 0.25; $CuSO_4{\cdot}5H_2O$, 2.36×10^{-4}; $ZnSO_4{\cdot}7H_2O$, 1.1×10^{-3}; $FeSO_4{\cdot}7H_2O$, 6.45×10^{-3}; and $MnCl_2$, 3.6×10^{-3}.

2.6. Citric acid fermentation

A. niger cells were activated by taking one vial of spore stock onto PDA agar plates and cultured for 72 h at 28 °C for *A. niger* SIIM M288. One loop of spores from PDA agar was streaked onto the PDA slant and cultured for 168 h at 28 °C. The spores from PDA slant were washed by sterile water and cultured in the seed medium for 36 h at 28 °C. Spores concentration in seed medium were counted with haemocytometer and controlled at $2–3 \times 10^5$ spores per milliliter of seed medium. The flask culture was carried out in a 250 mL flask containing 50 mL synthetic medium or corn stover hydrolysate with 10% (v/v) inoculation ratio at 28–37 °C, 200 rpm for 168 h. The initial pH was controlled by sodium hydroxide solution, in the consequent fermentation step the pH adjustment was not necessary. All experiments were done in duplicates.

The fermentor culture was carried out in a 3 L fermentor containing 1 L corn stover hydrolysate with 10% (v/v) inoculation ratio of *A. niger* SIIM M288 seed culture at 33 °C, 250–400 rpm, 1.0–2.0 vvm of ventilation volume for 8 days. The initial pH was controlled by 5 M sodium hydroxide solution.

2.7. Analysis of metal ions, sugars, citric acid and inhibitors

Elements of Zn, Fe, Cu, Mn, Mg, P and K in corn stover hydrolysate were determined using the inductively coupled plasma atomic emission spectrometry (ICP-AES). Prior to ICP-AES measurement, high sugar hydrolysate sample was digested by microwave-assisted acid digestion (Bizzi et al., 2011), then the metal concentration was analyzed using ICP-AES Agilent 725ES equipped with simultaneous CCD detector at 1.2 kW of power, 1.5 L/min of plasma gas flow, 1.5 L/min of auxiliary gas flow, 0.75 L/min of nebuliser flow, 15 rpm of pump speed, 35 s of sample delay time, 10 s of stabilization time.

Total nitrogen is determined by alkaline potassium persulfate digestion oxidation-UV spectrophotometric method according to Nydahl (1978). High glucose corn stover hydrolysate was digested at 120 °C of 30 min after diluted 10 times.

Glucose, citric acid, methanol, ethanol, acetic acid, formic acid and levulinic acid were analyzed using HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Japan) with Bio-Rad Aminex HPX-87H column at 65 °C. The mobile phase was 5 mM H_2SO_4 at the rate of 0.6 mL/min. Furfural, HMF, vanillin, syringaldehyde, 4-hydroxybenzaldehyde were analyzed using the reversed-phase HPLC (LC-20AT, UV/VIS detector SPD-20A, Shimadzu, Japan) with a YMC-Pack ODS-A column (YMC, Tokyo, Japan) at ambient temperature. The mobile phase was 50% acetonitrile solution at the rate of 1.0 mL/min. All samples were centrifuged to remove the cell mass and other water insoluble substances, then filtered through a 0.22 μm filter before analysis.

2.8. Citric acid yield calculation

The conversion yield of glucose to citric acid is defined as the ratio of the citric acid formed to the citric acid according to the theoretical value. The citric acid yield was calculated according the following equation:

$$\text{Citric acid yield} = \frac{[CA] \times V - [CA]_0 \times V_0}{[Glu]_0 \times V_0} \times \frac{M[\text{Glu}]}{M[\text{CA}]} \times 100\%$$

where $[CA]$ and $[CA]_0$ are the citric acid concentrations at the end and the beginning of the fermentation (g/L), V and V_0 are the volume at the end and the beginning of the fermentation (L), $[Glu]_0$ is the glucose concentration at the beginning of the fermentation (g/L), $M_{[\text{Glu}]}/M_{[\text{CA}]}$ (0.9375) is the conversion factor of glucose to citric acid on the basis of stoichiometric biochemistry.

3. Results and discussion

3.1. Tolerance evaluation of A. niger SIIM M288 to lignocellulose derived inhibitor

The influence of inhibitor compounds in corn stover hydrolysate on citric acid fermentation of *A. niger* SIIM M288 was evalu-

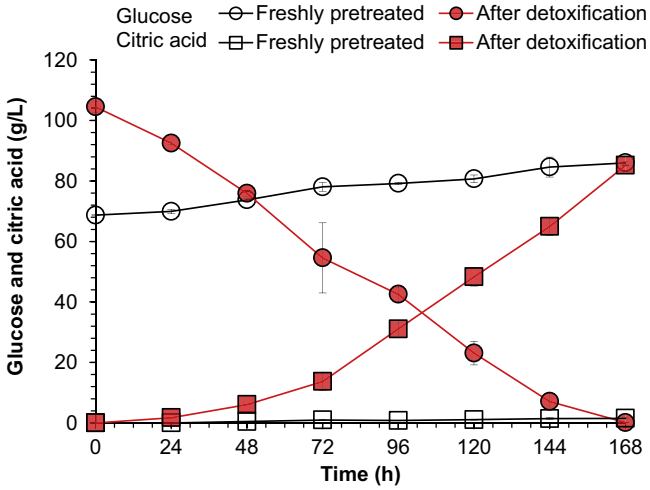

Fig. 1. Citric acid fermentation of *A. niger* SIIM M288 in the high glucose hydrolysate with and without inhibitor removal. Conditions: 33 °C, initial pH 6.0, 200 rpm, 10% (v/v) inoculation ratio for 168 h. "Freshly pretreated" indicates the corn stover hydrolysate prepared from the freshly pretreated corn stover without inhibitor removal step. "After detoxification" indicates the corn stover hydrolysate prepared from the pretreated corn stover after biodetoxification as described in Section 2.

ated (Fig. 1). When the hydrolysate was prepared using the freshly pretreated corn stover at 25% (w/w) solids loading, the hydrolysate contained 68.71 g/L of glucose and high inhibitor contents including 1.09 g/L of furfural, 0.84 g/L of HMF, 7.80 g/L of acetic acid, 2.46 g/L of formic acid, 1.43 g/L of levulinic acid, 0.43 g/L of vanillin, 0.20 g/L of syringaldehyde, and 0.02 g/L of 4-hydroxybenzaldehyde. After the pretreated corn stover was detoxified, the glucose of the biodetoxified corn stover hydrolysate at the same solids content (25%, w/w) was increased to 104.50 g/L from 68.71 g/L while the inhibitors were reduced to 3.50 g/L of acetic acid, 0.05 g/L of HMF, and no furfural was detected. No obvious citric acid generation was observed using the freshly pretreated

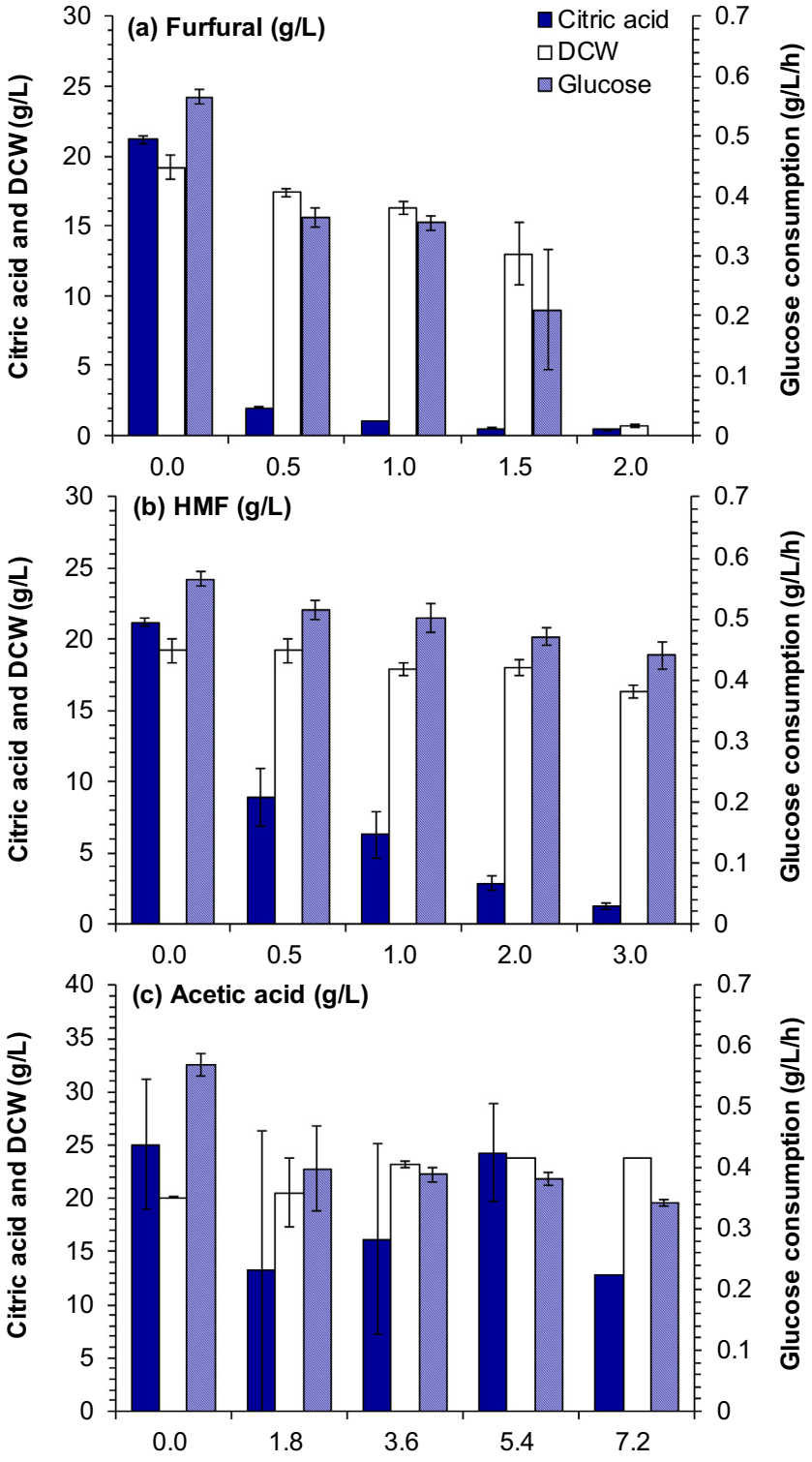

Fig. 2. Inhibitor tolerance of *A. niger* SIIM M288 during citric acid fermentation in synthetic medium. (a) furfural; (b) HMF; (c) acetic acid; (d) formic acid; (e) levulinic acid; (f) vanillin; (g) syringaldehyde; (h) 4-hydroxybenzaldehyde. Conditions: 28 °C, initial pH 6.0, 200 rpm, 10% (v/v) inoculation ratio for 168 h.

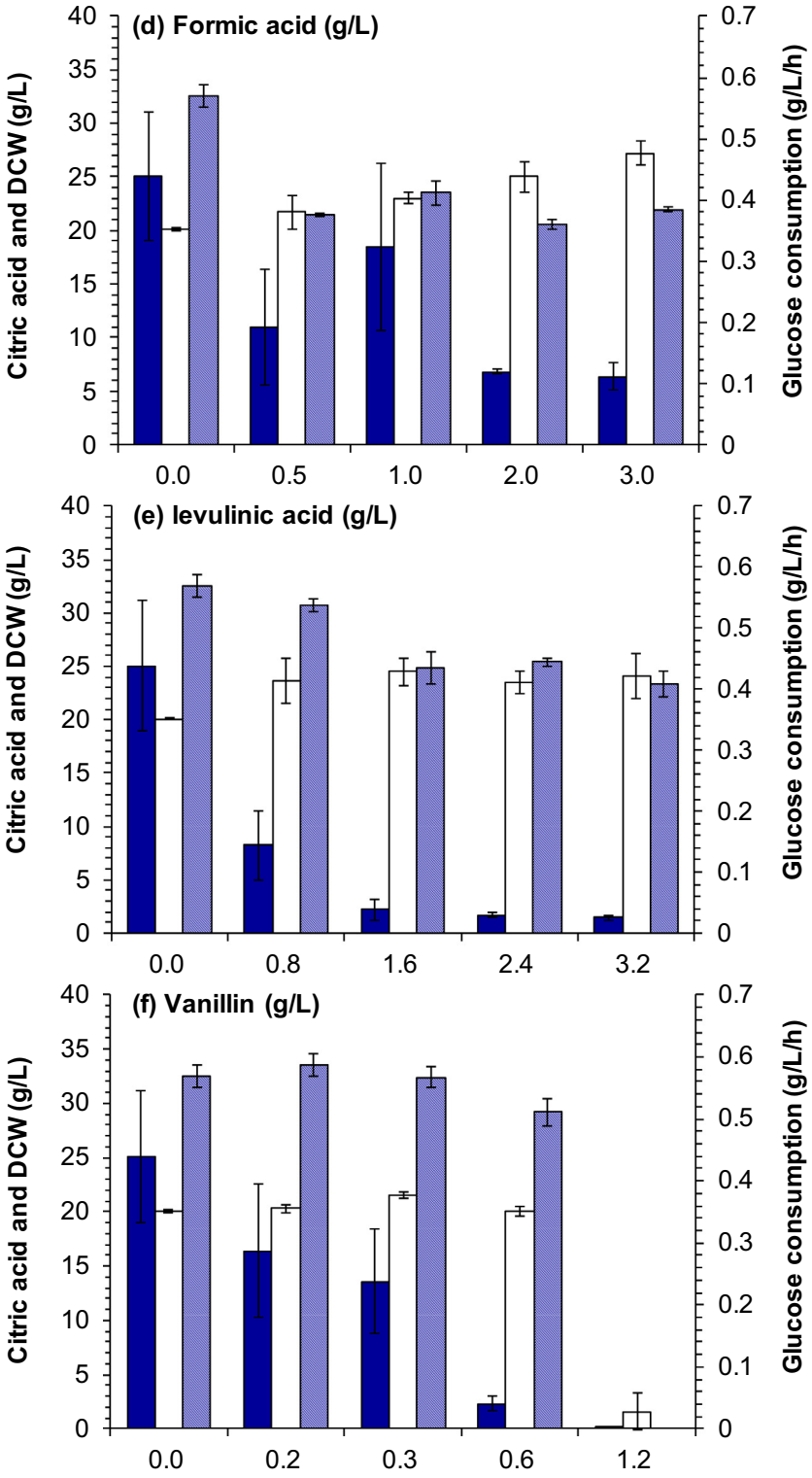

Fig. 2 (*continued*)

corn stover hydrolysate, while the citric acid titer was 85.18 g/L with the yield of 76.39% when using the biodetoxified corn stover hydrolysate.

The tolerance of *A. niger* SIIM M288 to each individual inhibitor were examined in details due to the importance of inhibitor existence on citric acid fermentation (Fig. 2). The selected eight model inhibitors included two furan aldehydes, furfural and HMF; three weak organic acids, acetic acid, formic acid, and levulinic acid; and three phenolic compounds, 4-hydroxybenzaldehyde representing *p*-hydroxyphenyl group compounds (H), syringaldehyde representing syringyl group compounds (S), and vanillin representing guaiacyl group compounds (G) (Palmqvist and Hahn-Hägerdal, 2000; Klinke et al., 2004). The concentration range of inhibitors was determined based on the measurement of inhibitors respectively in corn stover hydrolysate. Furfural showed the strongest inhibition on citric acid generation with the leading led to the

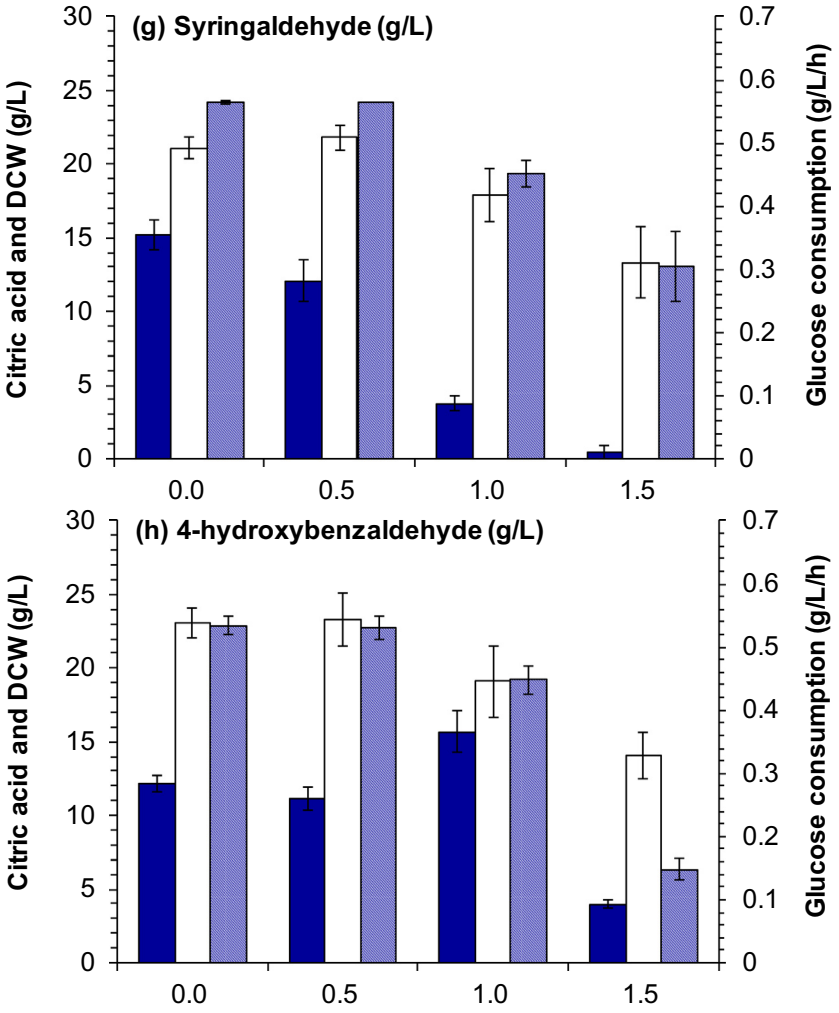

Fig. 2 (*continued*)

Table 1
Elemental analysis of corn stover hydrolysate and synthetic medium.

Elements	Synthetic medium (g/L)	Corn stover hydrolysate (g/L)
Nutritional elements		
N	2.5	0.47
P	0.72	0.17
K	0.57	0.12
Divalent metal ions		
Ca	0	1.7
Mg	2.4×10^{-2}	8.4×10^{-1}
Fe	1.3×10^{-5}	4.0×10^{-2}
Mn	1.6×10^{-3}	1.0×10^{-2}
Zn	2.5×10^{-4}	2.4×10^{-3}
Cu	6.1×10^{-5}	1.0×10^{-3}

High glucose hydrolysate was obtained by hydrolyzing 25% (w/w) of the pretreated and biodetoxified corn stover with the composition of 104.50 g/L of glucose, 22.86 g/L of xylose, 3.5 g/L of acetic acid, and 0.05 g/L of HMF. Furfural was not detected.

reduction of citric acid generation to only 15% of the control by the minimum furfural level of 0.5 g/L along with the considerable depressed cell growth and glucose consumption rate (Fig. 2a). HMF also showed the strong inhibition, although less than furfural, on citric acid generation by 54.17% reduction at 0.5 g/L of HMF with obvious inhibition on the cell growth and glucose consumption rate (Fig. 2b). Acetic acid inhibition on *A. niger* SIIM M288 was complicated and no significant change on its fermentability was observed even at the relatively high acetic acid level (7.2 g/L) (Fig. 2c). Formic acid inhibited citric acid generation, but the cell growth and glucose consumption rate were less affected (Fig. 2d). Unexpected, levulinic acid, generally regarded as a weak inhibitor, significantly reduced the citric acid yield (Fig. 2e). Vanillin significantly inhibited citric acid generation, but the cell growth and glucose consumption were almost not changed until vanillin concentration was very high (1.2 g/L) (Fig. 2f). Syringaldehyde was similar to vanillin: obvious inhibition on citric acid generation but less inhibition on cell growth and glucose consumption rate (Fig. 2g). Comparing to vanillin and syringaldehyde, 4-hydroxybenzaldehyde was a weaker inhibitor with less inhibition on citric acid generation, cell growth and glucose consumption rate (Fig. 2h).

A. niger SIIM M288 was highly sensitive to the existence of inhibitor compounds. Furfural was the most toxic one on citric acid fermentation, followed by HMF, formic and levulinic acid, and tolerant to relatively high level of phenolic aldehydes. The results suggest that the inhibitor removal step from corn stover hydrolysate was crucially important for *A. niger* to achieve high citric acid accumulation.

3.2. Nutrition abundance of corn stover hydrolysate on citric acid fermentation

Generally, citric acid fermentation medium is supplemented with nutrients such as phosphate, nitrogen, and inorganic salts

Table 2
Nutrients addition on citric acid fermentation performance in high glucose containing corn stover hydrolysate.

Nutrients	Amount (g/L)	Glucose consumption (g/L/h)	Citric acid titer (g/L)	Citric acid yield (%)
KH_2PO_4	0	0.55 ± 0.02	92.73 ± 4.03	81.79 ± 0.04
	2.5	0.55 ± 0.03	91.96 ± 4.98	87.38 ± 0.07
NH_4Cl	0	0.24 ± 0.01	36.64 ± 2.36	36.93 ± 0.03
	1	0.44 ± 0.08	74.62 ± 14.05	70.32 ± 0.13
	2	0.33 ± 0.01	55.49 ± 1.39	52.60 ± 0.02
	3	0.28 ± 0.01	47.62 ± 1.67	45.75 ± 0.02
	4	0.20 ± 0.01	33.54 ± 0.93	32.59 ± 0.01
$MnCl_2$	0	0.50 ± 0.03	84.01 ± 5.37	83.07 ± 0.07
	0.9×10^{-3}	0.52 ± 0.01	87.76 ± 1.59	86.34 ± 0.03
	1.8×10^{-3}	0.51 ± 0.01	84.91 ± 1.48	85.89 ± 0.02
	2.7×10^{-3}	0.52 ± 0.01	87.34 ± 2.27	85.65 ± 0.03
	3.6×10^{-3}	0.50 ± 0.01	84.45 ± 0.12	83.91 ± 0.04
	4.5×10^{-3}	0.52 ± 0.01	87.05 ± 0.60	86.68 ± 0.02
Full divalent ions	0	0.54 ± 0.01	90.44 ± 2.28	80.36 ± 0.02
	Full ions	0.43 ± 0.01	71.63 ± 2.53	65.96 ± 0.03

The glucose consumption rate was calculated in 168 h fermentation. The citric acid titer was measured at 168 h fermentation. The citric acid yield was calculated based on the equation as described in Section 2. The high glucose containing hydrolysate was prepared using the dry dilute acid pretreated and biodetoxified corn stover at the 25% (w/w) solids loading for enzymatic hydrolysis as described in Materials and Method section. The hydrolysate contained 104.50 g/L of glucose, 22.86 g/L of xylose, 3.5 g/L of acetic acid, and 0.05 g/L of HMF, no furfural was detected in the hydrolysate after the biodetoxification. Nutrients included KH_2PO_4, NH_4Cl, $MnCl_2$, and full divalent ions. The "Full divalent ions" indicates the addition of inorganic salts to the same composition in synthetic medium including (g/L): $MgSO_4{\cdot}7H_2O$, 0.25; $CuSO_4{\cdot}5H_2O$, 2.36×10^{-4}; $ZnSO_4{\cdot}7H_2O$, 1.1×10^{-3}; $FeSO_4{\cdot}7H_2O$, 6.45×10^{-3}; and $MnCl_2$, 3.6×10^{-3}. Fermentation conditions: 33 °C, initial pH 6.0, 10% (v/v)

for enhancement of citric acid accumulation. However, the elemental analysis showed that most of the nutrient salts were sufficiently enough in corn stover hydrolysate compared to that in the synthetic medium (Table 1) and the nutrient addition did not lead to even an average increase on citric acid accumulation (Table 2).

Phosphate and potassium contents in the hydrolysate were approximately 1/4 or 1/5 of the synthetic medium, respectively (Table 1). However, KH_2PO_4 supplementation to the level in synthetic medium (2.5 g/L) into the hydrolysate did not make any difference: 91.96 g/L with KH_2PO_4 addition and 92.73 g/L without KH_2PO_4 addition (Table 2). The result suggests that the phosphate and potassium contents in corn stover hydrolysate were already sufficient for citric acid fermentation.

Similarly, nitrogen in corn stover hydrolysate was 1/5 of the synthetic medium (Table 1), and nitrogen was supplemented by NH_4Cl addition (Table 2). Citric acid increased to 74.62 g/L from 36.64 g/L when 1.0 g/L of NH_4Cl was added, but then citric acid declined when NH_4Cl was increased from 1.0–4.0 g/L, suggesting that the nitrogen content in corn stover hydrolysate was not sufficient and the minimum addition was required.

Divalent metal ions are essential for the microbial growth (Angumeenal et al., 2002), but the major metal ions including Mg, Fe, Mn, Zn, Cu ions existed at high level in corn stover hydrolysate, approximately several orders of magnitude greater than that in the synthetic medium (Table 1). When the five metal ions were further supplemented into corn stover hydrolysate by adding $MgSO_4$, $FeSO_4$, $ZnSO_4$, $CuSO_4$, $MnCl_2$, citric acid accumulation was negatively affected: citric acid decreased from 90.44 g/L to 71.63 g/L with additional salts addition (Table 2). Although the Mn^{2+} deficiency is considered as the key factor on citric acid accumulation (Karaffa and Kubicek, 2003), the addition of Mn^{2+} to the hydrolysate in the range of 0–4.5 mg/L did not lead to any observable change on citric acid accumulation (Table 2). The results strongly indicate that the divalent metal ions in corn stover hydrolysate were already sufficiently enough and further addition may suppress the fermentability of *A. niger* for citric acid accumulation.

Summarizing the above result gives that corn stover hydrolysate already contained sufficient nutrients for citric acid fermentation by *A. niger* SIIM M288, including phosphate, potassium, and other divalent metal ions, thus no further supplementation of nutrients was needed. Nitrogen was slightly low and minimum addition of NH_4Cl was enough for high citric acid accumulation.

3.3. Optimizing fermentation parameters for high citric acid accumulation

Various fermentation parameters were optimized in flasks in the accepted range of industrial practice to obtain a high citric acid titer.

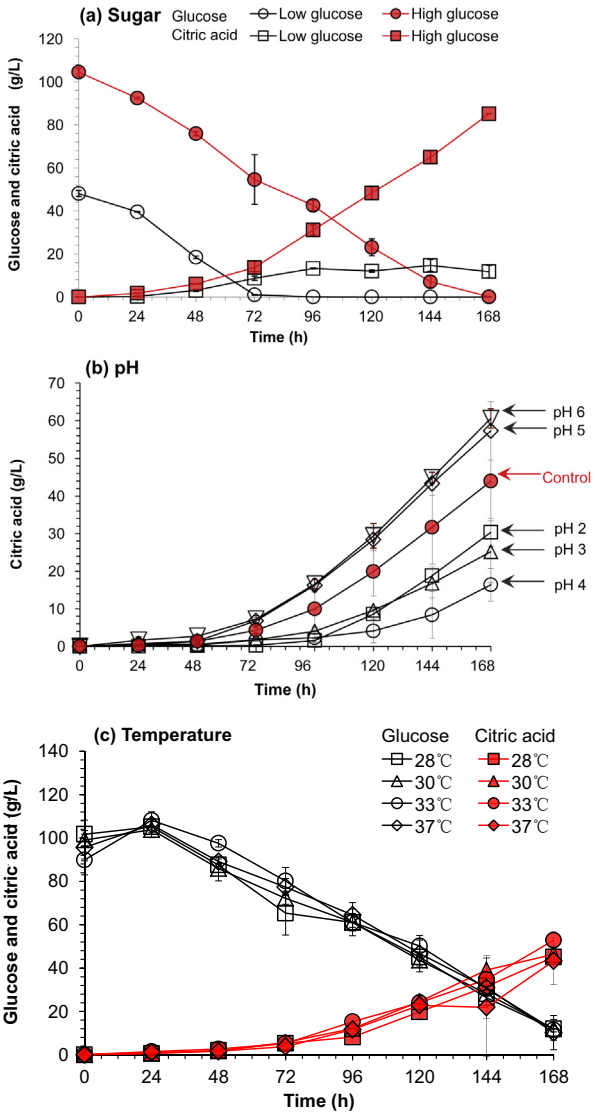

Fig. 3. Citric acid fermentation of *A. niger* SIIM M288 in corn stover hydrolysate under changing glucose concentration and initial pH. (a) Different sugar concentrations at initial pH 6.0; (b) different initial pH in the high glucose hydrolysate; (c) different temperature in the high glucose hydrolysate. Control indicates the pH of the hydrolysate was not adjusted. Conditions: 33 °C, 200 rpm and 10% (v/v) inoculation ratio. Control indicates the pH of the hydrolysate was not adjusted. Conditions: 33 °C, 200 rpm and 10% (v/v) inoculation ratio.

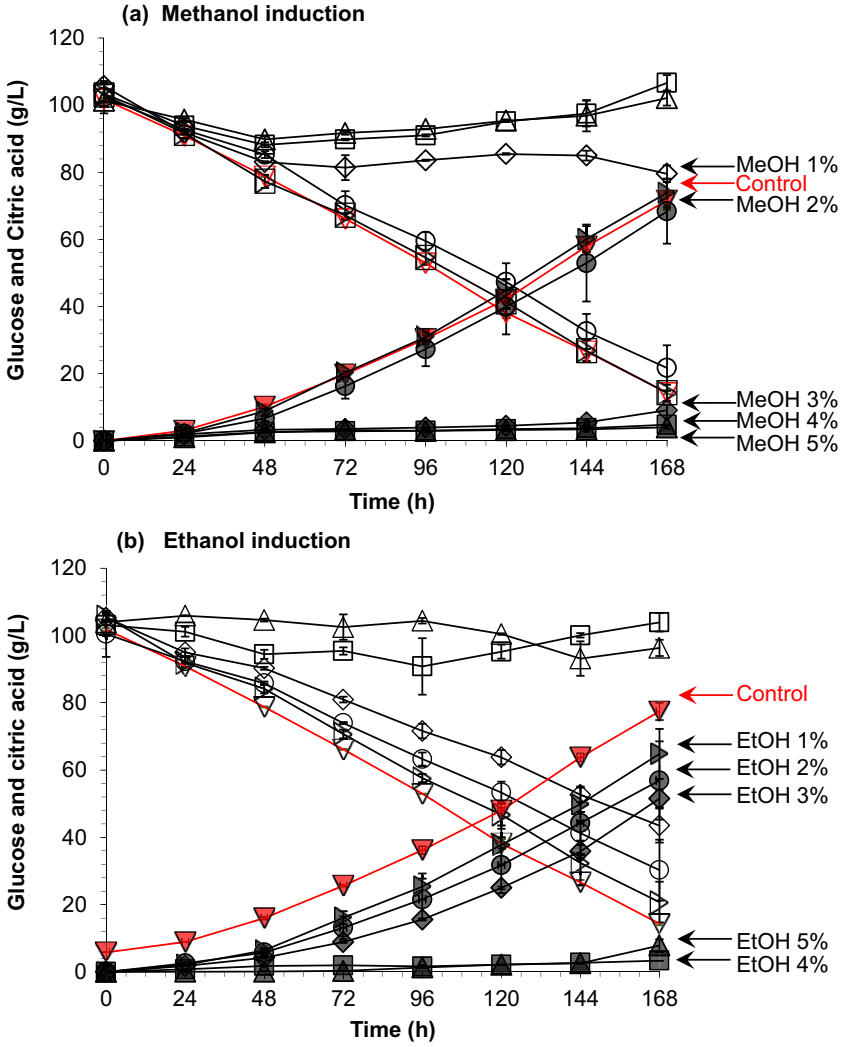

Fig. 4. Citric acid fermentation of *A. niger* SIIM M288 in corn stover hydrolysate under the induction of methanol or ethanol. (a) Methanol induction; (b) ethanol induction. Conditions: 33 °C, initial pH 6.0, 200 rpm, 10% (v/v) inoculation ratio. Open legends indicate glucose, and closed legends indicate citric acid. "Control" indicates the original hydrolysate without methanol or ethanol addition.

High sugar concentration is necessary to maintain a higher carbon-to-nitrogen molar ratio (C/N ratio) required for citric acid accumulation fermentation by *A. niger* (Gutiérrez-Rojas et al., 1995; Karaffa and Kubicek, 2003). Citric acid reached 85.18 g/L with the yield of 76.39% when glucose in corn stover hydrolysate was 104.50 g/L, but only 11.78 g/L of citric acid was obtained with yield of 22.97% when glucose in the hydrolysate was 48.05 g/L (Fig. 3a), suggesting the high initial glucose was the prerequisite condition of citric acid accumulation in corn stover hydrolysate.

Initial intracellular pH affects the metabolic flow of carbohydrates through glycolysis and the transport of citric acid across cell membrane (Angumeenal and Venkappayya, 2013). A relatively high initial pH of 5.0–6.0 gave the maximum citric acid in flasks (Fig. 3b). The most favorable temperature of *A. niger* SIIM M288 for citric acid fermentation in corn stover hydrolysate was 33 °C (Fig. 3c).

Inducers by methanol and ethanol are frequently used for increasing citric acid accumulations (Haq et al., 2003). However, but for the corn stover hydrolysate, citric acid accumulation in corn stover hydrolysate only slightly changed with methanol addition (1–2%) and further addition of methanol up to 5% negatively affected citric acid accumulation (Fig. 4a). Ethanol induction constantly decreased citric acid accumulation in the experimental range (Fig. 4b).

Citric acid fermentation of *A. niger* M288 using corn stover hydrolysate was further conducted in a 3L fermentor with accurate control of pH, temperature, and dissolved oxygen transfer. The hydrolysate was prepared by hydrolyzing 25% (w/w) of the dry dilute acid pretreated and biodetoxified corn stover solids. The fermentation was under 33 °C and 10% (v/v) inoculation of *A. niger* SIIM M288 seed. The pH was not regulated during the fermentation thus pH quickly decreased from the initial 6.0–2.5 after 48 h, then maintained relatively constant at 1.5–2.0. Citric acid reached 100.04 g/L with the conversion yield of 94.11% after 192 h fermentation (Fig. 5). Cell growth was also considerably high to 15.8 g of dry cell mass per L. As a comparison, citric acid titer and yield in flasks were 92.73 g/L and 81.79%. Up to our knowledge, this result is the maximum of citric acid fermentation from lignocellulose feedstock, almost three to ten folds greater than the maximum results of the similar studies in the previous publications.

An interesting phenomenon was repeatedly observed in the late stage of citric acid fermentation: the quickly generated citric acid did not match the slow consumption rate of glucose. This is most likely to be caused by the hydrolysis of oligosaccharides in corn stover hydrolysate because of the accumulation of glucoamylase secreted from *A. niger*. The glucoamylase is capable of oligomerizing glucose into α-linked di- and trisaccharides (Nikolov et al., 1989). Partial high titer glucose in corn stover hydrolysate was

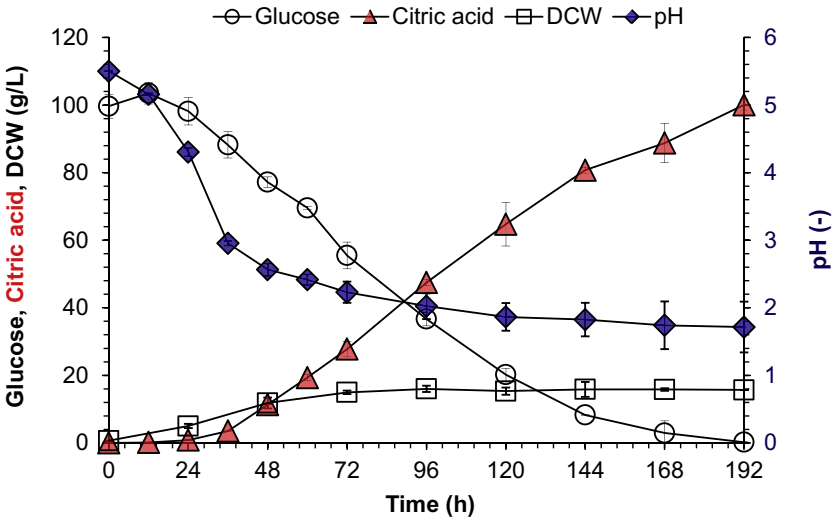

Fig. 5. Citric acid fermentation in 3L fermentor using corn stover hydrolysate prepared from dry dilute acid pretreated, biodetoxified and hydrolyzed at 25% (w/w) solids content. Conditions: 33 °C, initial pH 6.0, 10% (v/v) inoculation ratio of *A. niger* SIIM M288 seed culture.

converted oligomers by glucoamylase in the early stage of fermentation and then hydrolyzed into glucose in the late stage when the pH was maintained around 2.0 and glucose was consumed to a low level. Another reason is that the water insoluble calcium citrate formed in the early stage with the calcium ions in corn stover hydrolysate was solubilized in the late stage of very low pH environment.

4. Conclusions

Typical lignocellulose feedstock corn stover was pretreated by dry dilute acid method, biodetoxified, and hydrolyzed at high solids contents to obtain the high glucose hydrolysate. After fermentation condition and ion addition optimization, 100.04 g/L of citric acid titer and 94.11% conversation yield were achieved in fermentors fermentation with zero addition of extra nutrients and inducers in corn stover hydrolysate by *A. niger* SIIM M288, the nutrients concentration of corn stover hydrolysate were well satisfied with citric acid fermentation. This process prototype provided the potential for commercial application from lignocellulose feedstock.

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